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A Serum Cytolytic Factor Active against HeLa and Other Established Cell Strains.* (25393)

BERTIL BJÖRKLUND (Introduced by Maurice Landy)
Immunology Research Laboratory, State Bacteriological Laboratory and Radiumhemmet, Stockholm, Sweden

Immune lysis has invariably been associated with serum complement since its discovery more than 6 decades ago. Four components together with divalent cations are required for the activity of complement. More recently, evidence has been obtained that additional factors are involved(1). (In the present communication complement is understood to refer to the aforementioned normally occurring serum factors, which together with specific antibody bring about cytolysis and bactericidal and virucidal effects as well.) During investigations concerned with immunological properties of human tumor cells (2) the presence of a lytic system in normal serum differentiated from complement was revealed. This lytic system is present in serum as an inactive complex consisting of a cytolytic factor (CF) coupled with an inhibitor (CFI). Both of these factors have been prepared in a relatively pure state from human and from horse serum. It has been established that the serum cytolytic factor, under appropriate conditions, causes rapid and complete destruction of human tumor cells *in vitro*. This communication reports in summary form the characteristics of the cytolytic system and the methods by which its components are prepared and studied. The present work had its origin in studies of antigenicity of pooled

* This work was carried out under grants from Wallenberg Foundation and Swedish Cancer Soc.

malignant human tissue by means of cytotoxic reactions in cell cultures(2). Those studies clearly showed that independently of their origin(3), human cancer cells possessed in common an antigen linked to a lipoprotein(4) which was not demonstrable in normal tissues. The same antigenicity was also demonstrated on cells of the established atypical cell lines HeLa (Gey) and Detroit-6. Antiserum prepared in horses(2) immunized with antigen from pooled human carcinomas(2) displayed characteristic cytolytic effects on the aforementioned cell lines(2). The cytolytic activity remained undiminished following absorption with normal human tissues(2) but the activity was completely removed upon absorption with antigen derived from any of 35 individual human carcinomas(3). Indeed, these findings have since been extended to show that the specifically absorbing antigenic structures were present without exception in a total of 60 carcinomas.

Methods. Purified horse antibody to pertinent antigen of pooled human carcinomas was prepared by binding of the virtually insoluble antigen with excess antibody in the cold and eluting the antibody from the antigen under conditions of increased ionic strength and temperature according to the method used by Heidelberger and Kendall for purification of pneumococcus antibody(5). Other attempts to obtain antibody preparations were: precipitation according to Felton(6); Cohn fractionation (Methods 6 and 10)(7,8); zone electrophoresis(9); continuous curtain electrophoresis(10,11,12); chromatography on CMC and DEAE cellulose(13,14) as well as on calcium phosphate gel(15); precipitation with Na_2SO_4; by $(NH_4)_2SO_4$ and isoelectric precipitation. In these ways preparations were obtained with varying amounts of specific antibody, the activity of which was titrated in triplicate series of cell cultures. For details see(2,4). A common feature of the preparations was their ability to cause retraction and agglutination of monolayers of HeLa and Detroit-6 cells. Unfractionated immune serum not only had the same effect but also caused lysis of the cells(2). As the immune serum was fractionated to concentrate the antibody by removal of other serum constituents, the resultant preparations displayed less and less of the lytic effect. In fact, the most potent and purest antibody preparations, prepared by means of elution and continuous curtain electrophoresis,† were devoid of lytic activity. Cytolysis is known to require participation of serum complement. This seems to be true *e.g.*, for the actions of rabbit antisera to HeLa cells(16,17). Accordingly, it was anticipated that the addition of fresh serum to purified antibody would reconstitute the lytic system. This was found to be the case; addition of fresh normal horse or human serum to purified specific antibody consistently provided a system which was lytic for HeLa cells. The determining factors in the cytolytic reaction such as those of time, temperature, pH and concentration had been elucidated previously(2). This information was therefore applied and the test was standardized so that with antibody held constant the reaction was read as a function of the activity of the accessory lytic factors contributed by normal serum. Once the conditions for this reaction had been established the identity with complement of the lytic factors, contributed by fresh serum, was investigated.

Results. Serum which had been frozen at −20°C for 6 months and serum inactivated by heating for 45 min. at 56°C were equal to fresh serum in providing cytotoxic activity when combined with specific antibody. This undiminished potency of heat-inactivated serum has been observed in hundreds of tests. Actually the activity of serum withstood heating to 71°C for 30 minutes but 30 minutes at 73°C resulted in total loss of activity. Subsequently serum was separated into euglobulin and pseudoglobulin fractions; the C′1 and C′3 containing euglobulin was inactive while the pseudoglobulin, containing C′2 and C′4, carried all the activity. Treatment of serum with ammonia under conditions known to inactivate C′4 and with zymosan in a manner which effects removal of C′3(18), did not adversely affect the cytolytic activity.

While seeking to determine whether complement was involved in the cytolytic effect, the requirement for calcium and magnesium ions was examined. Normal serum was sub-

† Apparatus: Spinco Model CP.

jected to protracted dialysis against distilled water. Except for calcium and magnesium the salt concentration of the dialyzed serum was restored. Different amounts of calcium and magnesium were then added; these were without effect on the cytolytic events. However, when the concentration of calcium was increased to an amount well above that present in serum, cytolysis was totally suppressed. This effect was obtained also with barium and strontium but not with a considerable number of other divalent cations.

These findings clearly showed that the lysis of HeLa cells was caused by accessory factors in serum other than complement. Consequently a system or mechanism different from any hitherto described was operating to bring about cytolysis. The magnitude of the lytic effect was of such proportions as to render it inconceivable that the factor or factors responsible for final fragmentation of the cell could exist in serum in an active state. This is self-evident, from the fact that these cells normally are propagated in the presence of serum. It was therefore postulated that activation of the accessory mechanism involved release from the influence of an inhibitor. Such release was assumed to occur as a consequence of the antigen-antibody reaction known to take place on the cell surface. Antigen-antibody reactions are influenced by van der Waals' forces; a mutual partial discharge of the reacting molecules occurs, leading to a decrease of solubility. Inasmuch as many types of antigen-antibody reactions on the surface of cells lead to these same consequences it was visualized that changes in the physical, rather than the chemical state, were involved. The forces which release lytic activity from initially inert serums are therefore likely to be relatively weak, possibly involving shifts in colloidal-chemical equilibria. Application of physical forces such as an electrical field and/or changes of ionic environment might therefore effect the same changes. If this were the case, and if the lytic factor could be prepared in an active state freed from its inhibitor, one would expect that the lytic factor itself would produce lysis even in the absence of antibody. Whatever the ultimate explanation, it was considered worthwhile to attempt the separation of the active lytic factor and its inhibitor. Toward this end serum was subjected to continuous curtain electrophoresis. With M/150 phosphate buffer at a pH of 7.9 a fraction was obtained which migrated slightly faster than albumen. Of the 25-30 fractions collected from each run only this fraction was found to exert lysis of HeLa, Detroit-6, heart, and conjunctiva cells all of which are known to possess atypical (malignant) properties. However, the same fraction had no discernible effect on freshly established cultures of dog kidney and testes; monkey kidney (second generation); rabbit spleen suspension; and human and sheep erythrocytes before or after sensitization with isoantibody and amboceptor, respectively.

The isolated cytolytic factor (CF) could be further purified by electrophoresis. It was readily adsorbed on calcium phosphate gel, $BaSO_4$, kaolin, Bentonite, polyvinyl chloride, and on CMC and DEAE cellulose. However, attempts to elute CF from these adsorbents were unsuccessful. The activity of CF was suppressed by calcium ions at levels of 0.5-1.0 mg/ml. Heating to 73°C for 30 minutes destroyed CF, but 71°C for 30 minutes was tolerated. Addition of whole serum to CF stoichiometrically inhibited its cytolytic effect, presumably as a consequence of excess of reactive inhibitor.

CF could be inhibited completely by a single serum fraction (CFI) precipitated from whole serum by decrease in ionic strength to 0.01 at a pH of 7.9. This fraction was relatively homogeneous by electrophoretic criteria. An inactive complex consisting of CF and inhibitor (CFI) could be prepared by mixing the 2 fractions and allowing them to stand at 0°C for 20-40 hours. The inactive complex thus obtained exerted no cytolytic effect. However, addition of specific antibody to this inactive complex resulted in full cytolysis. The cytolytic system, as it was first observed with whole serum and specific antibody, could thus be reconstituted. This sequence of events in the interaction of antibody, cell, and the CF : CFI complex may be depicted as follows:

Schema for immune activation of CF

$$A + C \longrightarrow AC \text{ (intermediate)}$$
$$CF{:}CFI \longrightarrow CF + CFI$$
$$CF + C \longrightarrow \text{Lysis}$$
$$\longleftarrow Ca^{++}$$

Key: A = antibody; C = cell surface; CF = cytolytic factor; CFI = cytolytic factor inhibitor.

Specific antibody reacts with receptors on the cell surface resulting in a complex which, provided the cell exhibits active pinocytosis, is rapidly brought inside the cell. Thus the antigen-antibody complex is exposed only momentarily to factors present in the environment of the cell. The specific antigen-antibody reaction effects the release of CF from its native state of combination with inhibitor. The CF thus liberated at the cell surface causes a reaction, the results of which are immediate cessation of the movements of cytoplasmic granules and subsequent fragmentation of the cell. In the presence of Ca-ions, at levels well above the physiologic, CF no longer exerts these effects.

Many investigators have observed cytotoxic effects of serum on tumor cells; some of these observations extend back many years. In most instances, if not all, the heat-labile factors and the mechanism of the effect were not known. For the following reasons it is clear that the test system employed in the present work was not influenced by any of the previously reported serum activities: a) CF is not inactivated at 56°C; b) the cell strains used were propagated in a medium containing whole serum; c) whole serum by itself caused no lytic effect, indeed, serum suppressed the lytic effect of CF.

The interaction of CF and CFI in conjunction with the reactions of antigen-antibody has led to the recognition of a lytic system which had not been described heretofore. The information on CF as part of a biologic system is presently insufficient for the full interpretation of its significance. It is nonetheless apparent that a factor with these distinctive properties could have important implications for the reaction of the host to neoplasia.

Summary. Human serum was shown to contain a cytolytic factor coupled to an inhibitor. The lytic factor could be released by antibody specific to structures on the surface of human tumor cells. This factor could also be dissociated from its inhibitor by dialysis and obtained in a relatively pure state following electrophoresis. It then displayed a lytic effect on 4 established cell strains of human origin.

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Internal Antigenic Determinants in Protein Molecules.* (25394)

TERUKO ISHIZAKA,† DAN H. CAMPBELL AND KIMISHIGE ISHIZAKA‡
Division of Chemistry and Chemical Engineering, California Inst. of Technology, Pasadena§

For approximately 15 years, the problem of fragmentation of foreign antigens after injection, and the role of such fragments in immune responses has been of interest to us. Much of the original basic research work has been reviewed by Campbell and Garvey(1) and speculations and theories regarding the significance of such fragments have been presented by Campbell(2). If these antigen fragments play a role in immune mechanisms and antibody formation, then regardless of what theory of antibody formation one prefers, certain consequences can be expected. For example, one would predict that since the actual specific antigenic determinant is relatively a small part of the total molecule, antigen fragments should present determinants which do not occur on the surface of the native antigen molecule. Consequently, antibodies would be formed against internal structures of the native antigen molecule which do not occur on the surface of the native antigen molecule and would not be detected when antiserums were tested with the native intact antigen. Antibodies to such internal molecular structures ("hidden determinants") could be detected only by tests with partially degraded antigens. The problem is complicated by the fact that *in vitro* degradation might be different from that which occurs in the body and that simple inhibiting haptens might be formed. Lapresle and his collaborators(3) have shown that antibodies are produced against digested proteins which are not present in antiserums against native proteins, but the question arises as to the significance of the difference, and whether the difference in specificity is an artifact as a result of *in vitro* digestion. Probably the first evidence of "hidden" antigenic determinants on a molecular level was shown by Bartel and Campbell(4) using a dissociable hemocyanin as an antigen. Their data demonstrated that antiserums from rabbits immunized with hemocyanin contained antibodies which reacted with the dissociated molecular units, but not with the intact associated hemocyanin molecule. Our investigation was undertaken to see if an antigen such as bovine serum albumin (BSA) could be broken down *in vitro* into fragments which would react with rabbit anti-BSA serums that had been absorbed with intact native BSA. The results of these experiments are of a preliminary nature, but are very provocative and clearly suggest the reality of "hidden" valencies in immune mechanisms.

Materials. Our studies deal with crystalline BSA (Armour and Co.) and rabbit anti-BSA serums. Degraded BSA was prepared by digestion with crystalline pepsin (Worthington Biochemical Corp.) as will be described later. Anti-BSA serums were prepared by immunization of rabbits with BSA following 4 different schedules. The 6 rabbits of the first group were injected intravenously with 10 mg BSA in saline 3 times a week for 6 weeks. The 3 rabbits of the second group were immunized with the same antigen 3 times a week for 8 weeks. The 4 rabbits of the third group were immunized by intravenous injections 4 times a week for 4 weeks with increasing amounts of a neutral suspension of alum precipitated BSA. The 3 rabbits of the fourth group were immunized with BSA suspended in Freund's adjuvant, and 12.5 mg of protein was injected subcutaneously once a week for 8 weeks. These rabbits were bled 7 days after the last injection. The amount of anti-BSA in these antiserums, determined by quantitative precipitin analysis, is shown in Table I. The antibody against intact BSA molecules in these antiserums was absorbed with BSA. An amount of BSA corresponding to the equivalence point was added

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† Permanent address, Nat. Inst. of Health, Tokyo, Japan.

‡ Fulbright Fellow, premanent address, The Nat. Inst. of Health, Tokyo, Japan.

§ Contribution No. 2503.

TABLE I. Reactions between Absorbed Antiserums and Fractions of the Digested BSA.

Group*	Rabbit No.	Conc. of anti-BSA, mg/ml	Reactions with absorbed antiserums					
			Ring test			PCA		
			Fraction 1	2	BSA	Fraction 1	2	BSA
1	1	.93	+	—	—	—	—	—
	2	.78	+	—	—	—	—	—
	3	.24	+	—	—	—	—	—
	4	.21	+	—	—	—	—	—
	5	.41	+	—	—			
	6	.07	+	—	—			
2	1	.62	+	—	—	+	—	—
	2	.73	+	—	—	+	—	—
	3	.78	+	—	—	+	—	—
3	1	.58	—	—	—			
	2	.75	—	—	—			
	3	.83	—	—	—			
	4	.67	—	—	—			
4	1	1.18	—	—	—			
	2	.77	—	—	—			
	3	.73	—	—	—			

* Group 1 and 2, immunized with BSA in saline (see text); Group 3, immunized with neutral suspension of alum precipitated BSA; Group 4, immunized with BSA suspended in Freund's adjuvant.

to each antiserum. After 48 hours at 4°C, the specific precipitates were centrifuged off. The supernatants were tested for presence of anti-BSA and of BSA by ring test. The antibody was not detected in any supernatant, although a trace of BSA was present in some of them. These supernatants are the absorbed antiserums used in subsequent experiments. Purified anti-BSA was prepared by dissociation of specific BSA-anti-BSA precipitates(5). The gamma globulin fraction of anti-BSA serum was first removed by precipitation with ammonium sulfate, at ⅓ saturation (pH 7.8), and purified by repeated precipitation. The final precipitate was redissolved in saline, and dialyzed against borate buffered saline at pH 8.4. After dialysis the antibody was precipitated at the equivalence point with BSA. After 24 hours at 4°C, the precipitate was removed, washed 3 times with and resuspended in saline. The pH of the suspension was adjusted to 3.2. After shaking for one hour at room temperature to dissolve the precipitate, the insoluble residue was removed by centrifugation in a Spinco preparatory centrifuge at 78,000 g for one hour. The supernatant was fractionated with saturated sodium chloride at the same pH. The precipitate was dissolved in and dialyzed against a borate buffer of pH 8.4. Nitrogen content in the final preparation was 0.11 mg N/ml. About 60% of the total protein was precipitable with BSA at the equivalence point.

Results. Degradation of BSA and separation of antigen fragments reacting with absorbed antiserums. A degraded preparation of BSA was prepared by pepsin digestion. A 1% solution of BSA in saline was adjusted to pH 4.2, and 1 mg/ml of crystalline pepsin was added. The solution was allowed to stand for 24 hours at room temperature. To prevent further peptic digestion, the solution was heated to 58°C for 45 minutes and filtered to remove the coagulated protein. The solution was then adjusted to pH 8.6, and dialyzed against several changes of saline for 3 days at 4°C. The preparation was then layered on absorbed antiserums from Group 1 rabbits, and on normal rabbit serums as a control. After one to 2 hours white rings appeared at the interfaces with absorbed antiserums, whereas such a reaction was never observed with normal serums. On the other hand, it was found that the absorbed antiserums did not give any reaction with 0.01 to 10 mg/ml of BSA layered on the antiserums. These results suggested that anti-BSA rabbit serums contained some antibody which reacted with digested BSA, but not with intact BSA molecules.

In view of this result, and the fact that the digestion product was very heterogeneous, attempts were made to separate the solution of digested BSA by starch block electrophoresis and to study the various fractions. Zonal electrophoresis on a starch block was carried out in the usual manner, using barbital buffer (pH 8.6, $\mu = 0.1$). The solution of digested BSA was concentrated by pervaporation at 4°C and dialyzed against saline. Protein concentration of the preparations was about 3%. A volume of about 7 ml of each sample was allowed to migrate on starch for about 19 hours at 300 volts. The starch block was then cut into one cm segments, and each segment was eluted with 7 ml of saline. Each eluate was tested for protein concentration by the biuret reaction(6), and for the presence of antigen by ring test with the absorbed

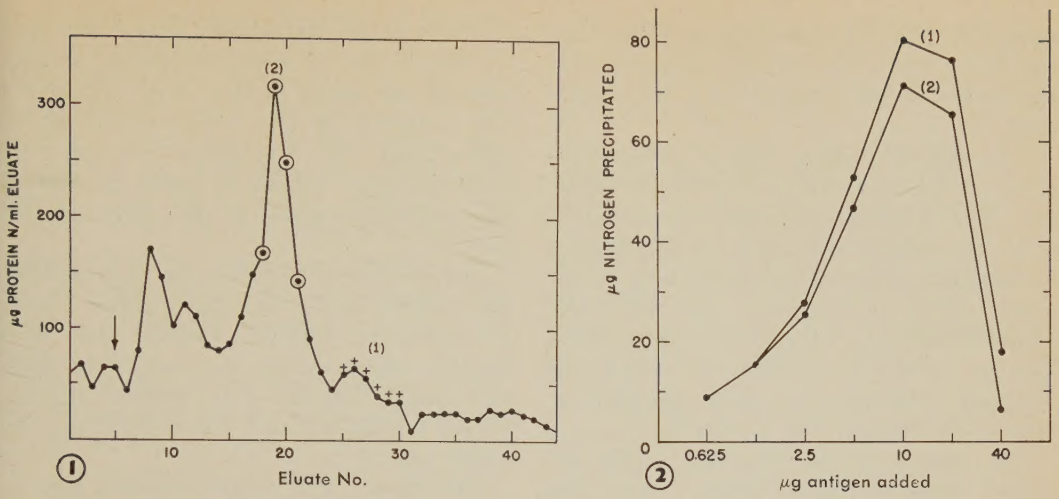

FIG. 1. Starch electrophoretic pattern of digested BSA. (1) represents Fraction 1 (No. 24 to No. 30). (2) represents Fraction 2 (No. 18 to No. 21). Plus represents presence of reactive substance(s) against absorbed antiserums. Arrow indicates the origin.

FIG. 2. Inhibition of the BSA-anti-BSA precipitin reaction with Fraction 2. Curve 1 represents precipitin curve between BSA and anti-BSA. Curve 2 represents precipitin curve in presence of Fraction 2.

antiserums of Group 1. A representative starch block electrophoresis pattern is shown in Fig. 1. Several eluates that had a mobility greater than the highest peak, definitely reacted with the absorbed antiserums. These eluates (from 23-30 cm) were pooled and designated as Fraction 1. The eluates from the segments corresponding to the highest peak were pooled separately and designated Fraction 2. By ring test, Fraction 1 gave a definite reaction with the absorbed antiserums, whereas it gave no reaction with either specifically purified anti-BSA or normal rabbit serum. To study the possibility that the reactive substance in Fraction 1 might be pepsin, heated pepsin was tested for reactivity with the absorbed antiserums. A saline solution containing 1 mg/ml of pepsin was heated at 56°C for 30 minutes, and 2-fold dilutions of the solution were layered on the absorbed antiserums. However, no positive ring tests were observed in any of the tubes, indicating that the reactive substance in Fraction 1 was not pepsin. The digestion of BSA and fractionation of the degraded material by starch block electrophoresis was repeated 9 times. The antigen fragments reactive with the BSA-absorbed antiserum were always detected only in the eluates from similar segments of the starch block, as described above.

In some preparations Fraction 2 contained materials that gave positive ring tests with specifically purified anti-BSA. In other preparations, however, Fraction 2 did not give reaction with the purified anti-BSA. It became apparent that Fraction 2 of these preparations contained substances which inhibited the BSA-anti-BSA precipitation. 0.25 ml volumes of Fraction 2 were added to equal volumes of anti-BSA serum. After 15 minutes at room temperature, 0.5 ml volumes of 2-fold dilutions of BSA were added and incubated for 48 hours at 4°C. The precipitates were centrifuged, washed 3 times with cold saline, and precipitated nitrogn was determined. The BSA-anti-BSA precipitates at the equivalence point, and especially in the region of antigen excess, were decreased by the addition of Fraction 2 (Fig. 2).

To get some idea of the properties of the reactive antigen in Fraction 1, the digested material, without further purification by starch block electrophoresis, was analyzed by free boundary electrophoresis, using barbital buffer (pH 8.6, $\mu = 0.1$). The mobility of the highest peak was -6.9×10^{-5} cm^2 volt^{-1}sec^{-1}, which is the same as the mobility of intact BSA. As the Fraction 1 migrates faster than the highest peak (Fraction 2) in starch block electrophoresis, the mobility of the reactive

antigen in Fraction 1 is probably faster than the intact BSA. This finding suggested that the antigen is the fragment of a BSA molecule.

Dependence of the presence of antibody reactive to Fraction 1 on immunization schedule. The absorbed antiserums from Group 1, 2, 3, and 4 rabbits were tested for the presence of antibody against the degraded BSA (Fraction 1 and 2) by ring test. The results are shown in Table I. All absorbed serums from Groups 1 and 2 gave definite reactions with Fraction 1, whereas none of the absorbed serums from Groups 3 and 4 reacted.

The gamma globulin fractions of the absorbed antiserums from Group 1 were also tested for presence of antibody. The gamma-globulin fractions were obtained by precipitation 3 times with ⅓ saturation ammonium sulfate (pH 7.8). A definite reaction was observed with Fraction 1, but not with Fraction 2.

As the first appearance of the reaction by ring test takes a long time (1 to 2 hours) as compared with the BSA-anti-BSA reaction, the passive cutaneous anaphylaxis (PCA) reaction and a complement fixation test were applied to detect antibody against the degraded substances. In the PCA reaction(7), guinea pig skin was sensitized locally with intradermal injections of 0.1 ml volumes of the absorbed antiserums from Groups 1 and 2. Three hours later these guinea pigs were injected intravenously with Evans blue followed by intradermal injections of 0.1 ml volumes of Fractions 1 and 2, 0.1% of BSA solution, and 0.9% saline as control into the sensitized areas. The guinea pigs sensitized with the absorbed serums from Group 1 did not give reaction with either the fractions of digested BSA or intact BSA, while the guinea pigs sensitized with the absorbed serums of Group 2 gave a definite reaction with Fraction 1.

Complement fixation tests were performed by the method of the U.S. Army Medical School, using veronal buffer containing an adequate concentration of calcium and magnesium ion(8) in place of saline. The absorbed antiserums of Groups 1 and 2 were tested using Fraction 1 and intact BSA (0.01 mg/ml) as antigens. Three series of 2-fold dilutions of the antiserums were made with the buffer, and an adequate amount of complement was added to each tube. Fraction 1 was added to the first series; to the second series intact BSA was added. To the third series an equal volume of buffer in place of the antigen was added as the antiserum control.

The complement fixation reactions between the absorbed antiserums and Fraction 1 were not definitely positive; however they were slightly different from the completely negative results obtained both between the absorbed antiserums and intact BSA or antiserum control.

Discussion. The results presented here indicate the presence of an antibody which reacts with degraded BSA but not with the intact BSA molecules in some of the rabbit anti-BSA serums. The reactive antigen in the degraded materials did not give reaction with the specifically purified anti-BSA, while the absorbed antiserums, which gave a reaction with the degraded substances, did not contain anti-BSA. These findings indicate that the specificity of the reaction between the degraded substances and the absorbed antiserums is quite different from that of the BSA-anti-BSA reaction.

The appearance of the precipitin reaction (ring test) between the degraded substances and the antiserums absorbed with BSA took a long time (1 to 2 hours), and the white ring on the interface was not as sharp as that of the BSA-anti-BSA reaction. The possibility arises that the degraded materials might react with serum protein nonspecifically, and thus give a reaction. This possibility is unlikely because the degraded substances gave no reaction with normal rabbit serums. Another possibility, which may explain the delay in appearance of the reaction, is the presence of inhibitors in the digested material. Although the reactive antigen was partially purified by starch block electrophoresis, there might be some inhibiting substances present, such as simple haptens in the fraction, and the precipitation might be inhibited by them. Moreover, the reactive antigen in the degraded material might not be the same fragment of BSA as that which would be present

in vivo, participating in formation of the antibody as a template. In other words, the reaction between the degraded protein and the absorbed antiserums might be a kind of cross reaction. These might be the reasons why the reaction was weak and took a long time to appear. The positive PCA reactions with Fraction 1, but not with either Fraction 2 or intact BSA in the guinea pigs sensitized with the absorbed antiserums, also suggest the presence of antibody against fragments of antigen.

The presence of antibody, directed toward the "hidden sites," in the antiserums does not parallel the concentration of anti-BSA (Table I). The antiserums prepared by immunization with soluble antigen reacted with Fraction 1, whereas the antiserums prepared with either the alum-precipitated antigen, or the antigen mixed with Freund's adjuvant did not, indicating that the presence of the antibody relates to the properties of the antigen injected, and/or the schedule of immunization. These findings suggested to us that the rate of destruction of antigen *in vivo* might be delayed by addition of the adjuvants, and that production of antibody against the internal structure of the antigen might have some relation to the process of antigen destruction.

Summary. 1. Positive ring tests and PCA reactions were observed between pepsin-degraded BSA and some of the BSA absorbed anti-BSA rabbit serums, suggesting the presence of the antibody against antigenic determinants which are not on the surface of the native protein molecule. 2. The presence of the antibody to the "hidden sites" did not parallel the concentration of anti-BSA in the serum but rather seemed to be related to the state of the antigen used in the immunization, and to the immunization schedule.

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Early Response of Plasma Volume, Red Cell Mass and Plasma Proteins To Massive Hemorrhage.* (25395)

MORTON D. PAREIRA, KENNETH D. SERKES AND STANLEY LANG
Surgical Research Labs., Jewish Hospital, St. Louis, Mo.

The response of blood volume and plasma proteins to massive hemorrhage in the rat has been reported(1). Measurements were made at 12, 24, 48 and 72 hours after bleeding. After hemorrhage of about 50% of measured blood volume, plasma volume was replenished within the first 12 hours. Arterial hematocrit was lowest at 12 hours, and although rising at 72 hours after hemorrhage, had returned to only 67% of control level by that time. Plasma protein concentration was fully restored by 48 hours, and was already 88.5% of control value at 12 hours, which was the earliest post-hemorrhagic measurement made. Since it was apparent that major plasma volume and protein concentration changes occurred prior to 12 hours, the experiments have been repeated with measurements of circulating volumes and plasma proteins at 2, 4, and 8 hours after hemorrhage. Such measurements were not made in the earlier study for 2 reasons. First, the purpose of that study was

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to find a relatively stable experimental preparation to evaluate tolerance to trauma after initial massive bleeding. In pilot studies after massive hemorrhage, maximum hemodilution occurred at 12 hours, and it was believed that 12 hours was suitable for earliest determinations of the measured parameters. Secondly, there was no assurance that radiochromium tagged cells would be suitably mixed for reliable volume studies in animals so recently in hemorrhagic shock. Our purpose here is 2-fold: 1) to report results of mixing time experiments to establish the validity of radiochromium tagged red cell dilution technic in animals in hypovolemic shock and 2) to report results of measurements of plasma volume, red cell mass and plasma proteins 2, 4, and 8 hours after massive, controlled hemorrhage.

Methods. Exp. I. To determine whether chromium 51 tagged red cell mixing was complete in animals in shock, so that measurements of red cell mass in such animals could be considered valid, the following experiment was performed. Rats were subjected to bilateral hind limb tourniquet application(1). Duration of tourniquet application was 4 hours, which produces a 75% mortality by 48 hours after tourniquet release. Rate of fluid accumulation in the hind limbs after tourniquet release is greater during the first 2 hours with a diminishing rate between that time and 4 hours. Three hours after tourniquet release, when animals were in profound hypovolemic shock, tagged red cells were introduced into the circulation and various mixing times were allowed before sampling for red cell mass determinations. The mean red cell masses in groups of animals so treated were compared with the mean red cell mass of a normal control group. *Exp. II.* Details of methods for determination of red cell mass, plasma volume and plasma proteins have been described(1). Male rats of Holtzman Farms weighing 240 to 320 g were used. Light ether anesthesia was used throughout. Controlled hemorhage was performed for 3 to 5 minutes after cannulation of the femoral artery with a polyethylene catheter (PE #10) attached to a Luer-Lok adapter and fitted to 10 ml syringe. In previous experiments the syringe contained approximately 0.1 ml of sodium heparin (10 mg/cc), injected into the artery prior to blood withdrawal. It was observed in those experiments that animals continued to ooze from the site of arterial cannulation after blood withdrawal until volume determinations were made. Since this introduced a slightly variable hemorrhage rather than a completely controlled one, bleeding procedure was modified. The syringes were merely wetted with heparin and any excess heparin was expressed through the cannula prior to cannulation. At end of bleeding period, the syringe was disconnected from the adapter and about 0.1 ml of protamin sulfate (1% solution) was injected intraarterially *via* the cannula. The artery was then ligated and the incision closed with skin clips. The wounds were inspected and were bloodless at time of volume measurements (2, 4, and 8 hours after hemorrhage). The controlled hemorrhage was 2.5 ml/100 g of body weight. Body weight, hematocrit, plasma protein concentration and red cell mass were determined in non-hemorrhaged control group and at 2, 4, and 8 hours after hemorrhage in experimental groups (one group of animals at each time). Blood volume, plasma volume and grams of circulating protein/100 g of body weight were then calculated for each determination.

Results. Exp. I. Reliability of the chromium 51 red cell tagged dilution method for measuring red cell mass in shocked animals is demonstrated by the fact that no difference is evident in the red cell mass as measured in tourniquet shocked animals when 15, 30 and 60 min are allowed for mixing of tagged cells, or between these values and those of control animals (Table I). The hematocrit of shocked animals is greatly elevated over hematocrit readings of non-shocked animals due to plasma loss in the former.

Exp. II. Plasma volume continued to rise following hemorrhage throughout period of observation (Table II). Significantly, it was 93% of control value at 2 hours, 101% of control at 4 hours and 108% at 8 hours after hemorrhage. Concomitant with this finding, the hematocrit reached its lowest value at 8

TABLE I. Measurements of Red Cell Mass in Tourniquet Shocked Rats.

Treatment	Wt	Hematocrit	Red cell mass	p†
Control (6)	216.3 ± 9.94*	46.5 ± 1.05*	2.57 ± .13*	
15 min. mixing (8)	206.2 ± 11.12	66.8 ± 2.28	2.60 ± .13	$p = .20$
30 " " (7)	193.3 ± 9.82	64.4 ± 1.93	2.51 ± .15	$.02 < p < .05$
60 " " (5)	197.9 ± 7.49	66.6 ± 1.34	2.56 ± .09	$.50 < p < .70$

* Stand. dev. † Comparison of means by Student "t" analysis. ( ) = No. of animals.

hours. Red cell mass showed no tendency toward replacement during period of observation.

Plasma protein concentration rose from its lowest level at 2 hours to reach 87% of control level at 8 hours. In spite of super-normal plasma volume at 8 hours total blood volume remained considerably below control value (81%) due to reduced red cell mass.

The calculated amount of plasma proteins/100 g of body weight, although less than the control value throughout the observation period, showed a considerable increase during the first 2 hours and in the last 4 hours of the experiment. Since approximately 50% of plasma protein was removed with the hemorrhage, the 73% of control value at 2 hours represents a sizeable inflow of protein as does the 92% of control value at 8 hours (as compared with the 73% at 4 hours).

Discussion. The present series of measurements at 2, 4 and 8 hours post-hemorrhage fits very well with previously recorded 12, 24, 48, and 72 hour series(1). From the earlier investigation it was not possible precisely to identify the time of greatest plasma dilution or lowest hematocrit level. This occurred between 8 and 12 hours. Value for the hematocrit at 8 hours was 64% of control and that of the 12 hour group 58% of control. The 24 hour hematocrit in the earlier study showed a rising value. Plasma volume is seen to have reached its maximum value by 8 hours and then remained stationary. The low point of hematocrit, therefore, coincides with maximum plasma volume and is uncomplicated by changes in red cell mass since there is little evidence of increase in red cell mass until 24 to 48 hours after hemorrhage.

There is no evidence for or against the possibility that sequestered red cells, not measured by the chromium dilution, are introduced into the circulation during hemorrhage or immediately thereafter. If such an event took place and was reversed sometime after hemorrhage (*i.e.*, when circulating volume was again adequate), a falling red cell mass might be evidenced in measurement sometime after the hemorrhage. The slightly lower red cell mass observed at 4 and 8 hours as compared with the 2 hour observation could be construed to support such a series of events. The theoreti-

TABLE II. Volume, Hematocrit and Plasma Protein Values in the Rat before and at 2, 4, and 8 Hours after Rapid Withdrawal of 2.5 ml Whole Blood per 100 g Body Weight.

Measured values				Calculated values		
Initial body wt, g	Hematocrit	Red cell mass, ml/100 g	Plasma proteins, g/100 ml	Plasma proteins, g/100 g	Total blood vol, ml/100 g	Plasma vol, ml/100 g
Control animals (N = 7)						
274.6 ± 17.3	48.3 ± 1.11 (100%)	2.52 ± .15 (100%)	6.34 ± .346 (100%)	.172 ± .008 (100%)	5.22 ± .21 (100%)	2.70 ± .15 (100%)
2 hr post hemorrhage (N = 6)						
279 ± 19.4	34.7 ± 2.42 (71.8%)	1.35 ± .07 (53.6%)	4.87 ± .15 (76.8%)	.126 ± .009 (73.3%)	3.90 ± .16 (74.7%)	2.55 ± .14 (94.4%)
4 hr post hemorrhage (N = 8)						
277.0 ± 12.8	32.0 ± 1.93 (66.3%)	1.30 ± .07 (51.6%)	4.59 ± .22 (72.4%)	.126 ± .016 (73.3%)	4.07 ± .39 (78.0%)	2.77 ± .22 (102.6%)
8 hr post hemorrhage (N = 9)						
280.1 ± 18.5	31.1 ± 2.50 (64.3%)	1.31 ± .10 (52.0%)	5.51 ± .22 (86.9%)	.158 ± .009 (91.9%)	4.22 ± .12 (80.8%)	2.91 ± .52 (107.8%)

cal remaining red cell mass following hemorrhage should have been 1.35 ml/100 g of body weight. This was the exact value found at 2 hours after hemorrhage. But it was 1.30 and 1.31 ml/100 g of body weight at 4 and 8 hours respectively after hemorrhage.

Plasma protein concentration forms a continuous function through the 2 series. In this series the plasma protein concentration was rising, reaching 87% of control value at 8 hours. Previously, it was 88.5% of control at 12 hours, 95% at 24 hours and equal to control value at 48 hours post-hemorrhage.

Total calculated protein content at 12 hours was 96% of control value. In the present study it was about 92% of control at 8 hours. The pattern of protein replenishment seems to have an early and late component. For example, the theoretical protein content after hemorrhage should have been 92 mg/100 g of body weight (control was 174 mg/100 g of body weight). At 2 hours post-hemorrhage it was 124 mg/100 g, a net gain of 32 mg/100 g or a 20% higher value than expected if no protein replacement had occurred. During the next 2 hours there was no change in total protein content, in spite of plasma volume gain. At 8 hours, however, the protein content had risen to 160 mg/100 g: an inflow of about 40 mg/100 g and a value 22% above that at 4 hours. This would seem to indicate a 2-phase plasma protein replacement, an immediate and a delayed one. The immediate plasma protein replacement is insufficient to restore concentration, while the slower process not only restores but exceeds the control level in terms of absolute amounts of plasma protein. The concentration does not, however, exceed the control value, suggesting a fine regulatory mechanism.

Summary. 1) The chromium 51 red cell tagged dilution method is valid for measuring red cell mass in the presence of hypovolemic shock as no difference was found in red cell mass measured in tourniquet shocked animals when 15, 30 and 60 minutes were allowed for mixing of tagged cells, or between these values and those of control animals. 2) The response of blood volume compartments and plasma proteins to acute withdrawal of 50% of circulating blood volume has been determined at 2, 4, and 8 hours after hemorrhage. Plasma volume is restored to 100% of the control within 4 hours and is 108% at 8 hours post-hemorrhage. Despite the elevated plasma volume, total blood volume remains well below the control, due to persistent red cell mass deficit. Changes in hematocrit inversely reflect plasma volume changes. Plasma protein concentration is restored to 87% of control by 8 hours. Protein replacement seems to occur in 2 stages. An immediate response is evident within the first 2 hours and a delayed response begins at 4 hours after blood withdrawal and extends over a 48 hour period.

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Response of Acutely Starved and Chronically Undernourished Rats to Saline Therapy Following Tourniquet Shock.* (25396)

KENNETH D. SERKES, STANLEY LANG AND MORTON D. PAREIRA
Surgical Research Labs., Jewish Hospital, St. Louis, Mo.

We previously demonstrated that a tourniquet trauma of sublethal magnitude in normal rats produced a mortality rate in acutely starved rats which correlated with degree of weight loss(1). Thus, there was 42% mortality in rats acutely starved to 80% of initial body weight and 100% mortality in rats acutely starved to 70% of initial body weight. A method for infusion treatment of tourniquet shock in the normal rat was later presented

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(2). This method consisted of slow administration of intravenous fluid over period of greatest fluid loss from the circulating volume. This slow uniform rate of infusion allowed achievement of higher survival rates at lower infusion volumes than had previously been reported in the rat. Survival rates were reported in normal rats at different levels of fluid replacement using normal saline, human plasma, 6% dextran in normal saline and 6% dextran in glucose in water. The present study is concerned with survival after saline infusion therapy in acutely starved and chronically undernourished rats with appropriate controls subjected to tourniquet shock.

Methods. Male albino rats of Holtzman Farms were maintained in air-conditioned laboratory on Purina rat chow and water *ad lib.* until start of experiments. Under each experimental condition, animals from each group served as untreated controls. *Exp. 1. Acute starvation.* (Saline infusion of 7 ml/100 g body weight.) Three groups of 16 animals each were used. At 9 a. m. on first day of experiment, 2 groups weighing 270-310 g had food withdrawn and at noon all animals were weighed to nearest ½ g on triple beam balance. This represents initial body weight. Group I (acute starvation group) received no food but water *ad lib.* until conclusion of experiment. Starvation period was 4 days, when their average was 78% of initial body weight. Group II (large control) was returned to cages and allowed food and water *ad lib.* Both groups were weighed daily thereafter. Group III (small control) animals continued food and water *ad lib.*; on day of tourniquet trauma their average weight corresponded to average weight of acutely starved group (*ca* 221 g). This group was intended to control any difference in response to therapy due to weight alone. Approximately 16 hours prior to application of tourniquets, food but not water was removed, (Groups II and III). On morning of experiment the animals were again weighed, anesthetized and bilateral hind limb tourniquets applied for 4½ hours. This duration of tourniquet trauma has been previously shown to be lethal to at least 96% of untreated controls in the 200-250 g weight range (2). At time of tourniquet release, animals were again anesthetized and a polyethylene catheter introduced into right jugular vein for administration of normal saline solution. Infusion was begun 1 hour after release of tourniquets, and rate of infusion so timed that 7 ml of solution was administered/100 g of body weight for 3 hours ± 17 minutes. Then the vein was ligated and the neck closed with skin clips. Animals were then returned to cages where food and water were available. Survival was recorded at 24 and 48 hours after tourniquet release(2). *Exp. 2. Acute starvation.* (Saline infusion of 15 ml/100 g body weight.) The experiment was repeated using this higher level of fluid replacement on each of 2 days. The results for each day and the combined results are indicated in the Table. *Exp. 3. Chronic undernutrition.* (Saline infusion of 7 ml/100 g body weight.) Three groups of 16 animals each were again used. *Group I* started in same weight range as in acute starvation experiments (260-310 g). Over 35 days they were given submaintenance amounts of Purina Lab. rat chow daily in such restricted amounts that at 35 days the animals were at 77.4% of their initial body weight (217 g). *Group II* (large control) also started in same weight range but allowed food and water *ad lib.* and at time of tourniquet trauma weighed 363.5 g. *Group III* (small control) was again a smaller group of rats allowed food and water *ad lib.* so that at trauma their average weight was in the same range as that of chronically undernourished group (215.9 g). All groups were submitted to 4½ hour bilateral hind limb tourniquet trauma. One hour following tourniquet release they received infusion of 7 ml normal saline/100 g body weight for 3 hours. Twenty-four and 48 hour survivals were recorded for all groups. Untreated controls were selected from each group. *Exp. 4. Acute starvation compared to chronic undernutrition.* (Saline infusion of 7 ml/100 g body wt.) A group weighing 260-310 g were placed on submaintenance diet of Purina rat chow so that over 35 days they gradually lost weight to approximately 80% of initial body weight. Four days before anticipated arrival of first group at the selected weight level, another group of equal size and in the same initial weight range was weighed

TABLE I. Survival in Acutely Starved Rats and Fed Controls following 4½ Hr Bilateral Hind Limb Tourniquet Trauma and Normal Saline Infusion of 7 ml/100 g Actual Body Weight (Exp. 1).

		24 hr			48 hr		
	Avg wt (g)	Lived	Died	Survival %	Lived	Died	Survival %
Group I (acute starvation)	%						
Treated	221.2 (77.9)*	3	8	27	0	11	0
Untreated	224.5 (77.8)	0	4	0	0	4	0
Group II (large control)							
Treated	282.9	11	0	100	11	0	100
Untreated	283.8	1	3	25	0	4	0
Group III (small control)							
Treated	241.1	11	1	93	9	3	75
Untreated	235.5	0	4	0	0	4	0

* % of initial body wt.

for initial body weight and thereafter given water but no food. On day of tourniquet trauma this group also weighed approximately 80% of initial body weight. On day of tourniquet trauma both groups were submitted to bilateral hind limb tourniquet application of 4½ hours duration. Beginning 1 hour after tourniquet release all animals were infused *via* right jugular vein with 7 ml normal saline solution/100 g body weight for 3 hours. Twenty-four and 48 hours survivals were recorded for both groups. Untreated controls were included for each group. The experiment was performed on 3 different days. Results for each day and combined results are indicated in Table IV.

Results. Infusions of 7 ml/100 g body weight of saline protected normally fed animals from death produced by tourniquet shock, but not animals acutely starved (Table I). When 15 ml saline/100 g body weight was given, survivors under acute starvation increased to 75% (Table II), while all treated control animals survived (Table II). Chroni-

TABLE II. Survival in Acutely Starved Rats and Fed Controls following 4½ Hr Bilateral Hind Limb Tourniquet Trauma and Normal Saline Infusion of 15 ml/100 g (Exp. 2).

		24 hr			48 hr		
	Avg wt (g)	Lived	Died	Survival %	Lived	Died	Survival %
Group I (acute starvation)	%						
Treated Day 1	209.8 (73.7)*	5	1		4	2	
" 2	231.8 (77.9)	5	1		5	1	
Total		10	2	83	9	3	75
Untreated Day 1	205.5 (73.7)	0	2		0	2	
" 2	226.2 (77.7)	0	2		0	2	
Total		0	4	0	0	4	0
Group II (large control)							
Treated Day 1	291	6	0		6	0	
" 2	289.7	6	0		6	0	
Total		12	0	100	12	0	100
Untreated Day 1	293	1	1		1	1	
" 2	298.5	1	1		1	1	
Total		2	2	50	2	2	50
Group III (small control)							
Treated Day 1	220	6	0		6	0	
" 2	221.2	6	0		6	0	
Total		12	0	100	12	0	100
Untreated Day 1	211	0	4	0	0	4	0
" 2	221.2	0	4	0	0	4	0
Total		0	8	0	0	8	0

* % of initial body wt.

TABLE III. Survival in Chronically Undernourished Rats and Fed Controls following a 4½ Hr Bilateral Hind Limb Tourniquet Trauma and Normal Saline Infusion of 7 ml/100 g Actual Body Weight (Exp. 3).

	Avg wt (g)	24 hr Lived	24 hr Died	24 hr Survival %	48 hr Lived	48 hr Died	48 hr Survival %
Group I (chronic undernutrition)	%						
Treated	217.8 (77.4)*	12	0	100	12	0	100
Untreated	227.1 (82.8)	3	1	75	2	2	50
Group II (large control)							
Treated	363.5	12	0	100	12	0	100
Untreated	369.4	4	0	100	4	0	100
Group III (small control)							
Treated	215.9	12	0	100	11	1	93
Untreated	223.9	1	3	25	1	4	0

* % of initial body wt.

cally undernourished animals under treatment with 7 ml/100 g dose had survival rate comparable to that of treated controls (Table III). Because of previously noted day to day variation in survival rates following tourniquet shock treated by infusion therapy(2), survival rate of animals acutely starved was compared simultaneously with that of chronically undernourished animals of same weight (Table IV). Higher survival rate of the latter was significant ($p<0.001$ by chi square).

Discussion. These experiments established that the response to tourniquet trauma followed by saline infusion therapy in chronically undernourished rats is as good as in normally fed rat in the same weight range. It also demonstrates that both of these groups show considerably better survival than acutely starved rats whose weight was reduced to the same degree.

The reasons for the difference are not clear. It could be suspected that the chronically undernourished group might have a relatively greater plasma or blood volume than the acutely starved group; however, we have previously shown(3) that at this level of body weight loss, total blood volume and plasma volume are higher in acutely starved than in

TABLE IV. Survival in Acutely Starved and Chronically Undernourished Rats following a 4½ Hr Bilateral Hind Limb Tourniquet Trauma and Normal Saline Infusion of 7 ml/100 g Actual Body Weight (Exp. 4).

	Avg wt (g)	24 hr Lived	24 hr Died	24 hr Survival %	48 hr Lived	48 hr Died	48 hr Survival %
Acute starvation	%						
Treated Day 1	231.8 (78.4)*	11	1		8	4	
" 2	242.3 (79.6)	8	5		4	9	
" 3	249.9 (79.8)	9	6		7	8	
Total		28	12	70	19	21	48
Untreated Day 1	240.7 (79.5)	0	4		0	4	
" 2	256.1 (80.4)	1	7		0	8	
" 3	256.3 (81.7)	0	5		0	5	
Total		1	16	6	0	17	0
Chronic undernutrition							
Treated Day 1	234.6 (80.3)	12	0		11	1	
" 2	229.6 (81.3)	13	1		10	4	
" 3	235.2 (83.6)	16	0		14	2	
Total		41	1	98	35	7	83
Untreated Day 1	237.0 (80.8)	4	0		3	1	
" 2	236.2 (83.7)	1	7		0	8	
" 3	233.2 (79.1)	1	5		1	5	
Total		6	12	33	4	14	22

* % of initial body wt.

chronically undernourished animal. Also, values for blood volume as percent of actual body weight were higher in the acute starvation group than the chronic undernutrition group. Serum protein concentration was essentially the same in both groups.

The unexpected survival of some untreated controls in the undernourished group also lends support to the conclusion that in some way chronic undernutrition allows a significantly higher survival rate after tourniquet trauma than does acute starvation of the same degree.

The difference in survival between acutely starved rats of Exp. 1 and 4, points to the variation inherent in such testing situations. We have previously demonstrated(2), in spite of rigid control of all obvious variables, there may be a sizeable day to day variation in survival. This is probably due in some degree to the relatively small numbers of animals which can be tested on any one day and the fact that death of 1 or 2 animals exerts a large effect. Comparison between groups must be made simultaneously on the same experimental day and enough days should be devoted to each experiment to produce significance of results.

Summary. Experiments were performed to ascertain possible differences in survival following tourniquet trauma and saline infusion therapy between rats chronically undernourished to 77.4-80.3%, and those acutely starved to 77-80% of initial body weight. It has been demonstrated that there is a significantly higher percentage of survival when chronically undernourished rats are infused with 7 ml normal saline solution/100 g body weight following an otherwise lethal tourniquet trauma. Acutely starved rats are not protected by this level of replacement, but do show 75% survival when infused at level of 15 ml saline solution/100 g body weight.

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A Rapid (24 Hour) Bioassay for Detection of Human and Mouse Tumor Factor. (25397)

F. FRIEDMAN, R. KASSEL, L. BURTON, M. L. KAPLAN AND A. ROTTINO
Hodgkin's Disease Research Laboratories, St. Vincent's Hospital, N. Y. City*

A cell free factor has been extracted and refined from human and mouse tumor tissue (1,2). This factor, when injected into C3H (f) mice will induce a wide variety of tumors, *i.e.*, parotid, mammary, and adrenal carcinomas; soft tissue tumors; and leukemia(1,2). For convenience, this factor has been designated as "Tumor Factor" (TF)(2). The method utilized for extraction and refinement of mammalian TF is the BFK (Burton-Friedman-Kassel) procedure(2). This procedure is an adaptation of the method originally developed for purification of the tumor inducing factor (TIF) present in the *tu-e* tumor strain of *Drosophila melanogaster*. Since both methods were closely related, past experience in the study of "*Drosophila* tumors" extending over a period of 10 years, suggested that mammalian TF could produce, in *Drosophila*, the lesion already described in the literature as "*Drosophila* tumor"(3-10,12). Preliminary data concerning the reaction of *Drosophila* to refined mammalian TF indicated that *Drosophila* could be used as an assay animal for rapid detection of mammalian TF. This report is concerned with a description of the technics developed from expansion of the preliminary observations.

Methods and materials. Drosophila hosts. The host line, *wild 51-52*, is a genetically sta-

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ble and hardy strain. Under standard environmental conditions, its survival rate is 90 to 95%. The dissection of more than 20,000 *wild 51-52* larvae and imagos, many of which were examined histologically, demonstrated that the *wild 51-52* line is a tumor free strain (7). No active TIF can be extracted from its larvae, pupae or adults(7). This strain is highly susceptible, however, to the tumor-inducing action of *tu-e* TIF (approximately 30 other strains of *Drosophila melanogaster* were tested for reactivity to the *tu-e* TIF; none of these strains had any appreciable reactivity). Tumor cells have never been observed in histological sections of *wild 51-52 Drosophila* larvae or adults which either had been injured or injected with control preps, *i.e.,* Waddington's salt solution, Tris (trimethylol aminomethane) hydrochloride buffer, extracts of *wild 51-52* larvae, dopa oxidase, tyrosine, tyrosinase, phenylalanine, phenol red, proteolytic enzymes (papain, carboxypeptidase, chymotrypsin, trypsin), lipase, amylase, and nucleases (DNAase and RNAase)(8,9,10). Since 96 hr old larvae are most reactive(7), only hosts of this age were used. Such hosts are acquired and maintained by standard technics(11). *Injection technics.* The injection apparatus was essentially similar to that described previously(7,12). However, the micro-injection needles were prepared as described by Kassel and Rottino (13), except that the maximum diameter at the base of the hypodermic bevel was 30 mu. Since the microinjection needles may be prepared rapidly by Kassel's technic, a new needle was used for each assay and used needles were immediately discarded. Host larvae were injected by refined injection procedures(12). All pre- and post-operative procedures and injection technics have been described(4,5,7-10). *Reading of Drosophila assay.* Twenty-four hours post inoculation, the host larvae were removed from the culture medium, washed with distilled water, and examined for presence of melanotic tumors. If present, the induced melanotic lesions(12) are readily detected under a binocular microscope at a magnification of 100 ×. The lesions induced by mouse and human extracts were found to be histologically similar to the tumor present in the *tu-e* spontaneous tumor strain(8) of *Drosophila. Preparations tested.* Preparations assayed for presence of TF were: a) TF refined by the BFK procedure from human material, *i.e.,* serum from Hodgkin's disease patients, Hodgkin's disease spleen and lymph node, buffy coat from myelogenous leukemic blood. b) TF refined from pooled liver, spleen, lymph node and brain of AK mice with spontaneous or transplanted leukemia and of C3H(f) mice with induced leukemia. c) TF refined from induced parotid tumors. d) Preparations, refined by the BFK procedure, of serum from a few "normal" individuals and 2 patients with non-neoplastic diseases. e) Crude serum preparations (BFK, Step 1)(1,2) of: pool of serum of 47 C3H(f) mice; patients with Hodgkin's disease, lymphosarcoma, lung cancer, mammary cancer and melanosarcoma. f) Inactive refined TF preparations, prepared by omission of high speed centrifugation in the BFK procedure(1,2). g) Tris buffer. Aliquots of refined TF, tested in *Drosophila,* were injected into our C3H(f) hosts. This host strain, methods of injection and tumors induced have been described(1,2).

Results. The application of the *Drosophila* assay (DA) technics to preparations obtained from mouse and human sources has indicated, to date, either the presence or the absence of active TF in these preparations. If present, TF activity in a given preparation is computed from the number of surviving larval hosts which had developed melanotic lesions within 24 hours after injection.

Results of assays, in both *Drosophila* and mouse, of refined mammalian TF are summarized in Table I. With one exception, all refined preparations which had TF activity in the DA, induced neoplasia in mouse hosts (Table I, parts A, B). Absence of active TF in preparations, as indicated by the DA, was confirmed by lack of tumor induction by these preparations in C3H(f) mice (Table I, parts B, D). The DA also did not detect any TF activity in the refined preparations from sera of 5 of 6 non-neoplastic human donors (Table I, part C). Both *Drosophila* and mouse assays indicated absence of TF activity in pro-

TABLE I. Refined BFK Preparations Tested.

	Drosophila assay			Mouse assay*
	Larvae inj.	Tumorous larvae/Surviving larvae	% induction	Tumorous animals/Surviving animals
A. Mouse source				
C3H(f)-AK—Leukemic tissue	150	19/125	15	22/37
Idem	133	17/115	15	5/9
AK—Leukemic tissue	127	16/111	14	4/12
Idem	140	4/112	4	1/6
Induced parotid tumor	135	9/114	8	4/29
B. Human source				
Hodgkin's disease serum	126	21/110	19	7/23
Idem, post-treatment	145	0/107	0	0/28
Hodgkin's disease R.B.C.'s	131	0/118	0	0/19
" " serum	700	33/565	6	7/29
" " spleen	160	4/128	3	0/26
" " lymph node	140	2/120	2	1/20
" " *idem*	173	14/142	10	1/14
Buffy coat from myelogenous leukemic blood	580	8/423	2	1/22
C. Serum from non-neoplastic donors†				
Nasal polyp	139	0/128	0	
Cirrhosis of liver	150	0/135	0	
"Normal"	147	0/122	0	
"	145	0/123	0	
"	141	0/117	0	
"	121	2/117	2	
D. Controls				
"Tris"	800	0/780	0	0/43
BFK prep., high speed centrifugation omitted	600	0/573	0	0/412

* No. of animals too small to compute % tumor induction.
† These preparations were not tested in mice.

cedural control preparations and Tris buffer (Table I, part D).

Crude BFK serum preparations of mouse and human TF induced formation of melanotic tumors in the injected *wild 51-52* larvae (Table II, parts A, B). These DA results (Table II), indicated that crude BFK preparations of pooled serum from 47 mice of our C3H(f) (spontaneous tumor-resistant, 1,2) line did not contain active TF, whereas active TF was present in Crude BFK serum preparations of cancer and Hodgkin's disease patients.

Discussion. The use of larvae of the *wild 51-52* strain of *Drosophila* as an assay animal has, among others, the advantages of: a) precise genetic control; b) rapid indication of end results and c) ease of handling and maintenance. The rapid (within 24 hours) "tumor" formation in the injected *51-52* larvae may have been stimulated by the physiological reactions occurring in the hosts during this period. Some of these physiological reactions

TABLE II. Crude BFK Preparations Tested.

	Drosophila assay		
	Larvae inj.	Tumorous larvae/Surviving larvae	% induction
A. Mouse source			
C3H(f) pooled serum	208	0/110	0
B. Human serum			
Hodgkin's disease	227	32/153	21
Idem	167	9/81	11
"	150	5/129	4
"	162	69/125	55
"	190	34/94	36
Lymphosarcoma	138	6/82	7
"	133	14/107	13
Leukemia	143	19/106	18
Lung cancer	151	16/127	12
Idem	129	17/103	17
Mammary cancer	145	1/86	1
Melanosarcoma	131	21/97	21

occurring are: (1) protein synthesis and histolysis of tissue (protein breakdown), with resultant liberation of amino acids; (2) increased nucleic acid metabolism; (3) increased titer and activity of growth and differentiation hormone; (4) increased feeding activity of the animal. Thus, increase in general larval metabolism, high level of protein synthesis and amount of amino acids in larval hemolymph, peak nucleic acid metabolism, and presence of large amounts of nutrients in larval hosts at time of TF incubation may well have collaborated to stimulate the rapid rate of "tumor" formation(9,10). These physiological reactions which possibly contribute to rate of "tumor" formation, may also be involved in production of tumors in the larval host injected with crude BFK preparations of mouse and human material. Thus, the substances present in crude preparations which maintain species specificity(1,2) do not appear to affect the reactivity of the tissue of injected *wild 51-52* larval hosts to the inductive capacity of mammalian TF. This phenomenon is in direct contrast to the lack of tumor formation obtained following injection of crude human preparations into mice.

Refined preparations of both mouse and human TF were used to correlate the DA with tumor induction in the mouse. Although the relationship appears to be too crude to allow quantitation at this time, a low DA has been confirmed by comparatively low induction rate in the mouse and conversely, a comparatively high DA was also confirmed by relatively high induction rate in the mouse. The results further indicate a positive correlation between a preparation's lack of TF activity in the DA and inability of this preparation to induce tumor formation in the mouse.

The importance of these results lies in the fact that for the first time the "*Drosophila* tumor" has been induced with extracts from human and mouse tumor tissue. Whether or not tumor factor preparations from different sources are biochemically the same is a matter for future research. Nevertheless, the simplicity of acquiring crude BFK preparations of human TF coupled with the responsiveness of *wild 51-52* hosts to these crude preparations may permit an eventual epidemiological survey for prevalence of TF in the serum of *Homo sapiens.*

Summary. An assay system has been described for detection, within 24 hours, of tumor factor isolated from mouse and human neoplastic tissues by the BFK technic. Attempts have been made to correlate the tumor factor activity in the *Drosophila* assay with tumor induction in mouse hosts.

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Some Properties of Fluorocarbon-Treated Animal Tissue.* (25398)

P. G. Stansly and D. S. Ramsey (Introduced by Bjarne Pearson)
Detroit Inst. of Cancer Research, Detroit, Mich.

This report is concerned with changes occurring in tissue subjected to extraction with fluorocarbon, trifluorotrichloroethane, CCl_2F-$CClF_2$. Although this type of solvent is finding increasing use in separation of viruses and viral constituents from host tissue, relatively little is otherwise known about the constitution of such extracts, and the changes they undergo at successive extraction stages. Some of our results in exploring the possibility of applying the procedure to aid in detecting and concentrating mouse tumor agents will also be indicated.

Methods. A 25% homogenate of tissue in 0.04 molar versene at pH 7 was homogenized for 5 minutes at high speed in a VirTis "23" apparatus with ½ its volume of trifluorotrichloroethane in the cold. After centrifugation at 2000 × g for 5 minutes, the turbid aqueous phase was removed and again homogenized with ½ its volume of solvent. The process was repeated for 5 or more extraction cycles. Protein, RNA and DNA were determined on the aqueous phase at each stage. An aliquot was centrifuged at 120,000 × g for 1 hr, and these constituents again determined on the supernatant to obtain sedimentable components. The final aqueous phase was concentrated by dialysis against polyvinylpyrollidone, then against 0.05 M phosphate, pH 7, and examined in the analytical ultracentrifuge at 196,000 x g at a 1% protein concentration. RNA was determined by the orcinol method, DNA by the indole reaction, and protein by the biuret procedure, using appropriate standard curves.

Results. As extraction proceeded, the homogenate was gradually depleted of its protein, RNA and DNA, the amount removed varying with the constituent and the preparation. An illustration is given in Fig. 1. In this case, acid insoluble RNA had already disappeared by the fourth extraction stage, whereas appreciable amounts of protein and DNA remained to the end. In general, protein was the one constituent which withstood removal by the solvent even after 7 extrac-

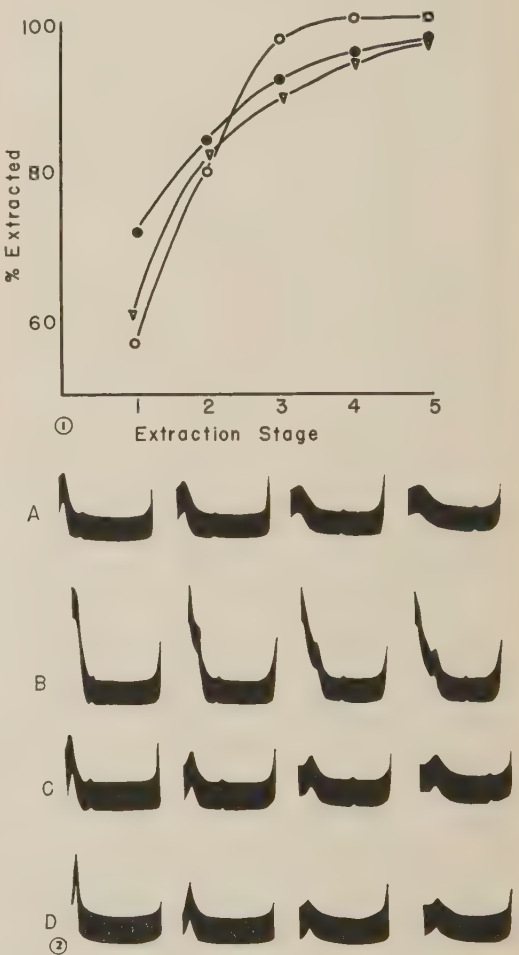

FIG. 1. Total RNA, DNA and protein in aqueous phases of fluorocarbon extracts of C3H mammary carcinoma at different extraction stages. ○ RNA, ● DNA, △ protein.

FIG. 2. Sedimentation curves of stage 5 fluorocarbon extracts in .05 M phosphate, pH 7. A, B, C and D are as indicated in Table II. Photographic exposures were at 8 min. intervals after a gravitational force of 196,000 × g was reached in a Spinco Model E centrifuge. Sedimentation constants of A, B and C were 23.4, 38.1 and 37.2, respectively.

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TABLE I. Sedimentable* Components in Fluorocarbon Extracts of the Mouse Mammary Tumor at Successive Extraction Stages.

Component	% sedimented at extraction stage 0	1	2	3	4	5
Protein	55	32	19	19	10	9
RNA	81	68	27	0		
DNA	96	91	87	87	78	70

* 120,000 × g for 1 hr.

tions. The terminal protein may be a fairly pure component. Thus, 67% of the protein of the Ehrlich ascites cells remaining after 5 extractions migrated as a single peak at pH 8.8 in the Tiselius apparatus.

Protein, RNA and DNA existed in both sedimentable and non-sedimentable forms in early extraction stages, whereas in advanced stages, one or more of the components were entirely unsedimentable, as for example, RNA at stage 3 of Table I. The RNA remaining at this stage was completely degraded, as determined by its solubility in cold 0.25% uranic acetate in 2.5% perchloric acid, thus accounting for its behavior. However, this was not the sole explanation for part of the RNA being non-sedimentable in earlier extraction stages, as in stage 2, where 73% of the RNA was non-sedimentable, but only 58% acid soluble. None of the non-sedimenting protein or DNA was acid soluble.

Fluorocarbon extracts of 3 mouse tumors and of normal mouse kidney were concentrated and centrifuged at 120,000 × g for one hour. The results are shown in Table II. The differences in sedimentable RNA and DNA of these tissues are noteworthy. In the analytical centrifuge, a rapidly sedimenting component, representing about 5% of the total material, was observed in all 3 tumor preparations (Fig. 2), but absent from the normal mouse kidney. Similar preparations, derived from mixed viscera of normal mice of various strains, of their individual organs, and of whole fetuses, also failed to exhibit a sedimenting component of this type.

Discussion. Appearance of a unique particulate component in the 3 tumors, which was absent from normal tissues, is of particular interest. This substance was not observed in extracts before fluorocarbon treatment, possibly because it was obscured by normal tissue constituents. It is tempting to consider that this material may be of viral origin inasmuch as all 3 tumors probably have a viral component in their etiology. This is, of course, well established for the mammary carcinoma; the PL leukemia is in all respects analogous to the better known AK leukemia, and is therefore likely to be a viral neoplasm; the thymic tumor arose in a 9 month old C3H mouse that had been inoculated *i.v.* at 2 mos. with a cell-free PL leukemic extract, and would also therefore appear to be a viral neoplasm. This possibility is strengthened by the observation(unpublished) that fluorocarbon extracts of transplants of this tumor exhibit virus-like particles having an average diameter of 120 mμ.

The low sedimentation constants of these components (Fig. 2) suggest that they are not complete viruses. For example, the mammary tumor agent is reported to be several hundred S units(1), but the corresponding component we observed is only 38.1 units. Conceivably, the estimated 95% impurity could have strongly influenced the observed value. An alternative explanation is that the component was an incomplete virus(2).

It must be pointed out that the analytical data for RNA and DNA (Table II) do not clearly distinguish normal tissue from tumor tissue. But certain inferences may nevertheless be drawn. Thus, if the mammary agent is

TABLE II. Sedimentable* Components of Concentrated Stage 5 Fluorocarbon Extracts.

Exp.	Tissue	Conc. factor	Component	Amt sedimented μg/ml	Amt sedimented %
A	C3H thymic tumor	16	Protein	5000	23
			RNA	210	4
			DNA	0	0
B	C3H mammary tumor	3.4	Protein	2600	30
			RNA	0	0
			DNA	70	53
C	PL leukemic lymph nodes	2.7	Protein	1100	22
			RNA	55	7.2
			DNA	60	5.6
D	Normal mouse kidney	11.7	Protein	1320	13.9
			RNA	.8	1
			DNA	16	18.9

* 120,000 × g for 1 hr.
† From an inbred Swiss (Tumblebrook) strain.

resistant to extraction by the fluorocarbon, as appears to be the case(3), then it is more likely to be a DNA than RNA virus, for there was no intact RNA in this particular preparation. Similarly, the fluorocarbon extract of the thymic tumor had no DNA, hence was more likely to be a RNA virus. The leukemic tissue had both RNA and DNA but by inference, only the RNA would be expected to contain a viral component.

Summary. RNA, DNA and protein are differentially removed by extraction of a tissue with fluorocarbon CCl_2F-$CClF_2$. These constituents occur in sedimentable (120,000 x g, 1 hour) and non-sedimentable forms. A unique component was observed in concentrated fluorocarbon extracts of 3 neoplastic tissues, which was absent from normal tissue. Its possible relation to viruses is discussed.

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Distribution Studies on Radioactive Hg^{203} Sodium Mercaptomerin in Animals. (25399)

BENJAMIN CALESNICK, ARTHUR WASE AND VINCENT R. ALTARELLI
(Introduced by M. J. Boyd)
Depts. of Pharmacology and Biochemistry, Hahnemann Medical College, Philadelphia, Pa.

Many previous studies on mercurial diuretics have been performed on compounds administered clinically orally(1,2), or parenterally when combined with theophylline(3). This investigation was undertaken with Hg^{203} mercaptomerin sodium because, as employed clinically, it is not combined with any agent which might influence distribution or excretion. This study was done to determine general distribution, renal concentration, and rate of urinary excretion of this compound.

Materials and methods. Hg^{203} mercaptomerin sodium was synthesized in our laboratories,* and was identified by paper chromatography, quantitative determination of Hg and S, determination of temperature of decomposition, and determination of diuretic activity. The solutions were freshly prepared with pyrogen-free water and resterilized by filtering through type H. A. millipore filter. Specific activity of original material was 360/ μc/mg mercury when assayed for gamma radiation. A standard dose of 1 mg Hg/kg (equivalent to 3.5 mg/kg mercaptomerin sodium) was used. Thirty-three albino rats (165-190 g), injected intraperitoneally, were divided into groups of 3 and placed in metal metabolism cages. Each group was deeply anesthetized with ether and sacrificed 15, 30, 60, 90, 120, 150, 180, 210, 240, 270, or 300 minutes after injection of the radioactive compound. Blood was obtained by cardiac puncture, using heparin as anticoagulant, and both kidneys were removed, decapsulated, weighed, and digested in concentrated nitric acid. Average radioactivity of renal tissue is expressed as μg of mercaptomerin sodium/g wet weight of tissue. Aliquots from pooled urines of 60, 120, 130, and 240 minute groups were collected and assayed for radioactivity. In addition, a series of kidney sections of the 1, 2, 3, and 4 hour groups was prepared for radioautographs. Sections 8 μ thick of kidney tissue were prepared by usual paraffin impregnation technic and mounted without removal of paraffin. The slides were covered with Kodak Panchromatic XX photographic film and firmly fastened with tape. Optimum exposure time was 14 days. In a second study, Hg^{203} mercaptomerin sodium was injected in-

* Method of synthesis supplied by Wyeth Inst. for Medical Research, Radnor, Pa.

PLATE 1. Radioautographs of rat kidney following intraper. inj. of Hg^{203} mercaptomerin sodium (1 mg/Hg/kg).

tramuscularly into 28 albino rats (165-190 g) to study duration of radioactivity after a single injection. Fourteen groups of 2 animals each were kept in metabolism cages and a group sacrificed hourly for the first 3 hours, daily for the next 7 days, and finally weekly for the next 4 weeks. Twenty-four hour urines were collected for first 7 days. Plasma, renal tissue, and urine were assayed for radioactivity.

Results. Radioautographs (Plate 1) revealed that radioactivity was concentrated in renal cortical areas. Similar localization has been demonstrated after administration of radiomercuric chloride(4).

Intraperitoneally injected Hg^{203} mercaptomerin sodium (Fig. 1) produced a peak plasma concentration in 15-30 minutes and a peak renal accumulation in 30-60 minutes. On the other hand, when Hg^{203} mercaptomerin sodium was injected intramuscularly, peak plasma concentration occurred in 30 minutes and peak renal accumulation was 60 minutes (Fig. 2). Peak renal accumulation following intraperitoneal administration was approximately 25% of that produced by intramuscular injection. Wet weight of the rat kidney was 940 $\pm$ 26 (S.E.) mg.

We then calculated percentage of original material excreted in urine. The amount of mercaptomerin sodium recovered in urine during 4 hours following intraperitoneal injection was 301.1 μg or 42% of original dose. On the other hand, total amount recovered 24 hours following intramuscular injection was 41.2% of original dose. Only 78% of administered dose was recovered during first 72 hours and none was detected after 96 hours.

Additional studies were carried out on 2 apparently healthy male mongrel dogs, weighing 7.5 and 9.0 kg. These animals were anesthetized with pentobarbital sodium (30 mg/kg) and hydrated with normal saline by continuous intravenous drip at 0.1 ml/kg/min. throughout experiment. Renal venous samples were withdrawn through a polyethylene catheter introduced into left renal vein through an abdominal incision. Arterial blood samples were withdrawn through an indwelling needle in the carotid artery. Urine samples were collected with a urethral catheter introduced into the left side through the abdominal incision. Hg^{203} mercaptomerin sodium was injected into the femoral vein within 15 seconds. Blood samples were collected in syringes moistened with heparin. At 5, 10, 15, 30, 60, 90, and 120 minutes, samples were taken simultaneously from renal vein and carotid artery. Urine was collected at 10, 30, 60, 90, and 120 minutes. Arterial plasma concentration at the 5 minute period was only 5.8 μg/ml with an A-V difference of only 0.8 μg/ml (Fig. 4).

Discussion. Radioautographs indicate that mercury is most concentrated in the cortical areas with limited amounts in medulla of kidneys. This is in accord with the findings of Greif *et al.*(1) and de Metry and Aikawa(5). The radiopurity of Hg^{203} mercaptomerin sodium was ascertained by paper chromatography but no studies were performed to determine the chemical stability of this material in tissues. Although the locus of mercurial diuresis is presumably in the tubules, it has not been settled whether it is in the proximal or distal portion(6). However, all portions of the renal tubular structures are present in the region of highest radioactivity.

Radioactivity counts on whole kidney revealed that 91.7% of the injected radioactivity was present within one hour after intramuscular injection. This confirms the findings of Borghgraef *et al.*(2) and Weston *et al.* (7) who reported that mercury is rapidly taken up by the kidney. These data suggest that radioactive mercurial compounds are removed mainly by the renal cortex and are excreted into the urine. Delay in onset of excretion of mercury may be related to the time required to accumulate a critical concentra-

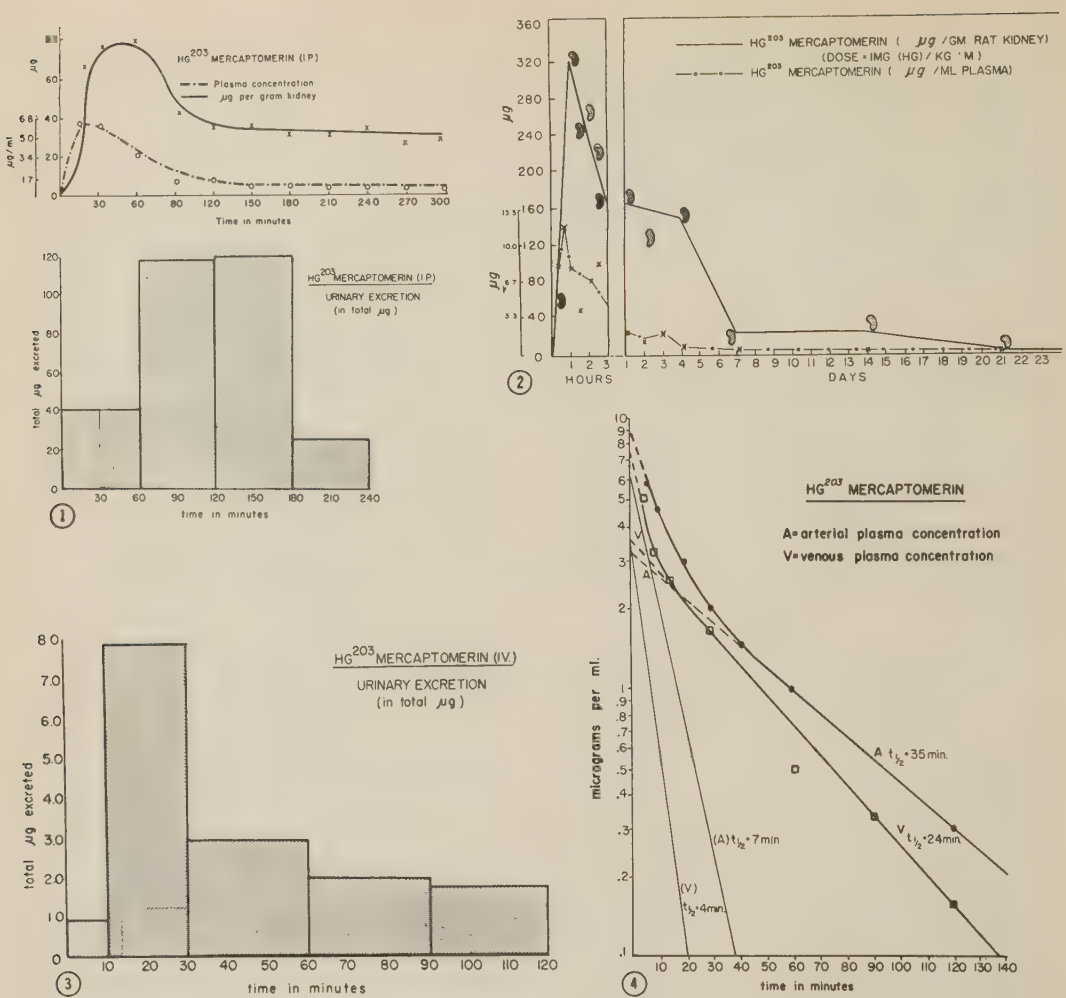

FIG. 1. Concentration of Hg^{203} mercaptomerin sodium in rats following intraper. inj. Urine values represent avg of 3 animals.

FIG. 2. Concentration of Hg^{203} mercaptomerin sodium in rats following intramusc. inj. Each "x" and each "kidney" represents avg of 2 observations.

FIG. 3. Urinary excretion pattern of intrav. Hg^{203} mercaptomerin sodium into dogs.

FIG. 4. Biological decay of plasma concentration following intrav. administration of Hg^{203} mercaptomerin sodium into dogs.

tion of this drug within the tubular cells. Following intravenous administration into dogs, renal excretion of Hg^{203} mercaptomerin sodium was most rapid during the first 30 minutes (Fig. 3) when the A-V difference was greatest (Fig. 4). Renal excretion decreased when the A-V difference diminished and approached a plateau between 90 and 120 minutes. The biological half-life, measured by rate of disappearance of radioactivity from plasma of arterial blood and venous renal blood, indicated at least 2 phases. Graphic resolution of the biological decay curves (Fig. 4) indicated the fast-moving component to have a half-life of 4 minutes for venous plasma and 7 minutes for arterial plasma. The slow-moving component has a half-life of 24 minutes for venous plasma and 35 minutes for arterial plasma. Similar regression curves were obtained by Borghgraef, Kessler, and Pitts(2) following intravenous administration of chlormerodrin.

Although peak plasma concentration and renal accumulation of Hg^{203} mercaptomerin

sodium, when administered intraperitoneally and intramuscularly, occurred at similar time intervals, intramuscular plasma concentration was approximately twice intraperitoneal concentration, and renal accumulation following intramuscular administration was 4 times that of the latter. This may indicate that renal tissue possesses a tremendous avidity or binding capacity for mercurial compounds.

Summary. 1. Using Hg^{203} labeled mercaptomerin sodium and studying radioautographs and renal distribution, 91.7% of an intramuscularly injected dose of 1 mg Hg/kg was found in the kidney within the first hour, and maximum concentration occurred in the cortical area. 2. Radioactivity was detected for 28 days in plasma and in renal tissue. 3. Intravenous administration of radioactive mercaptomerin sodium resulted in a rapid decline in plasma concentration during the first 5 minutes. Rate of urinary excretion appears to be a function of arterial plasma-renal vein plasma difference.

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In vitro Response of Mouse Testis to Human Chorionic Gonadotropin.* (25400)

ARDIS J. LOSTROH† (Introduced by M. E. Krahl)
Lab. D'Embryologie Exptl. du Collége de France, Paris

A number of investigators have cultured the mammalian testis successfully, either as entire organ or as fragments thereof(1-5). Irrespective of the medium employed or duration of culture, it was concluded that testicular explants had continued to develop in an essentially normal manner. These results are in contrast to those which I have obtained with ovarian explants from young post-natal mice in which an atrophy of the follicular and thecal cells occurs leading to a general loss of organization of the tissue; maintenance of ovarian structure *in vitro* was dependent upon incorporation of proper hormones into the basic medium(6). The following studies with isolated testis were therefore undertaken, using the same medium as that employed for the post-natal ovary, to determine response of the testis under these conditions. Human chorionic gonadotropin (HCG) and lactogenic hormone were the hormones selected for study.

Procedure. Testes from mice of the C57 Black strain, 3 days of age, were cultured on a medium made of 2 parts agar,‡ 1 part Tyrode's solution enriched with equal quantity of synthetic medium 199(7), and 1 part chick embryo extract. Details of the method have been discussed(8), and preparation of hormone-enriched medium has been described (6). Explants were cultured at 31 ± 1°C; every other day they were displaced to new sites on the media. In earlier experiments, the whole testis was cultivated 7 days on a medium enriched with lactogenic hormone or with HCG. In later experiments, each testis

* The author expresses appreciation to Prof. Etienne Wolff for a most rewarding year in the Lab. D'Embryol. Exp., and to Dr. C. H. Li and Leo Pharmaceutical Co. for hormone used.

† Fellow of National Foundation '57-58. Present address: Dept. of Physiology, Univ. of Chicago, Ill.

‡ Agar preparation containing 1 g agar (Difco)/100 cc Gey's solution.

was cut into 4 pieces and arranged around a fragment of ventral prostate; the explants were transferred to new media after 4 days and the experiments were terminated after 14 days. Tissues were fixed in Bouin's solution, embedded in paraffin and sectioned at 6 μ. The lactogenic hormone (30-35 I.U./mg) was prepared by Dr. C. H. Li(9); the HCG (1000 I.U./mg) was a product of Leo Pharmaceutical Co. Hormones were dissolved in Tyrode-medium 199 solution and incorporated into the basic medium in concentration indicated; 0.10 cc of the hormone-enriched medium was then added to each embryological watch glass in which a mixture of 6 drops agar, 3 drops Tyrode's solution and 3 drops extract had already congealed.

Results. The testis of the mouse, 3 days after birth, was too large to be cultured as a whole organ without occurrence of necrosis at its center; tissue at the periphery survived. On the other hand, fragments of testis were maintained without necrosis but there was a tendency toward tubular disorganization; a similar phenomenon was observed by Champy (1) with testicular explants from rabbits. At this stage of development the spermatogonia were distributed at the periphery of the tubules among future sertoli cells, and interstitial cells with a well developed cytoplasmic body were abundant (Fig. 1A). Representatives of each cell type persisted under *in vitro* conditions. Nonetheless, in 7 days of culture tubular size decreased, tubules came to lie in close proximity to one another, and interstitial cell cytoplasm was reduced (Fig. 1B). In 4 experiments it was then demonstrated that the inclusion of HCG in the basic medium ameliorated consistently the degenerative changes; however, it did not maintain the testicular morphology evident prior to culture (Fig. 1C). Especially relevant is the fact that, although the interstitial cells could be distinguished by their eosinophilic cytoplasm, the cytoplasmic body of these cells was not maintained.

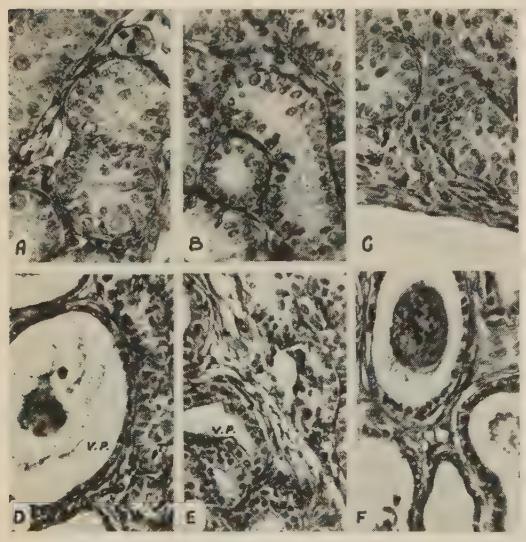

FIG. 1. A-C. Sections of gonads taken from male mice 3rd day after birth and cultured on hormone-enriched media for 7 days. 192×. D-F. Sections of ventral prostate taken from mice 27 days of age and cultured on hormone-enriched media for 14 days. 176×. Stain, hematoxylin-eosin; thickness, 6 μ.

A. Normal onset control. B. Testis cultured on medium enriched with lactogenic hormone, 1.25 mg /cc. C. Testis cultured on medium enriched with HCG, 250 I.U./cc. Cytoplasm of interstitial body distinguishable and eosinophilic. D. Explant cultured in association with testis on medium-enriched with lactogenic hormone, 1.25 mg/cc. No evidence of androgen production by testicular fragment. T = testis, VP = ventral prostate. E. Explant cultured in association with testis on medium enriched with HCG, 1250 I.U./cc. Note stimulation of prostatic epithelium. F. Explant cultured on medium enriched with HCG, 1250 I.U./cc.

Finally, to establish whether or not an enhancement in production of androgen could be effected by HCG under *in vitro* conditions, a target organ specific for the male hormone was cultured with the testis; fragments of one gonad were associated with a fragment of ventral prostate taken from a male mouse, 27 days of age. Figs. 1D and 1E demonstrate that testicular explants continued to secrete androgen for a period of 14 days when cultured on a medium enriched with HCG but not when cultured on a medium enriched with lactogenic hormone. In the absence of the testis, HCG was ineffective (Fig. 1F).

Discussion. Degenerative changes observed in the testis after 7 days of culture are modest. These results are in contrast to those previously obtained with ovarian explants taken from mice of the same age(6) and suggest that the testis is not as intimately dependent upon pituitary hormones as is the ovary during this period of development. The fact that embryonic testes can continue to secrete

androgen in an isolated system lends support to this interpretation(10).

Previous investigations have shown that anterior pituitary explants have a morphological influence on the isolated testis(5,11). The present study demonstrates that HCG has a similar effect on testicular explants and can stimulate production of androgenic hormone under *in vitro* conditions. Thus, as in the case of the ovary, the results obtained with the isolated testis are similar to those previously observed in experiments with the whole animal(12).

Summary. Testes from mice, 3 days of age, were cultured in an *in vitro* system for 7 or 14 days on a medium composed of agar, Tyrode's solution, synthetic medium 199 and chick embryo extract. Degenerative changes in control explants included a decrease in size of tubules and a reduction in cytoplasm of interstitial cells. Inclusion of HCG in the medium ameliorated these changes. Moreover, measurable amounts of androgen were produced by testes cultured on media enriched with HCG whereas no detectable amount was produced by those cultured on media enriched with lactogenic hormone.

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Studies on the Small Particle Complement-Fixing Antigen of Foot-and-Mouth Disease Virus. (25401)

JEROME POLATNICK (Introduced by M. S. Shahan)
Plum Island Animal Disease Lab., Agric. Research Service, U. S. Dept. of Agriculture, Greenport, N. Y.

It has been well established that foot-and-mouth disease is associated with at least 2 specific particles(1,2). The larger particle, reported to be 23 mμ in diameter and having a sedimentation coefficient (s-rate) of 140, possesses all the viral properties of the infection together with about half of the complement-fixing activity; the smaller particle, 8-10 mμ in diameter and having an s-rate of 14 displays complement-fixing (CF) activity only (3,4). Preliminary chemical investigation of the smaller particle, hereafter to be called the CF-antigen, indicated a globulin nature for the material(5). This report presents the results of biochemical studies on the CF-antigen and purification procedures.

Materials and methods. Virus infectivity was measured by a plaque assay using primary bovine-kidney cell layers in 4-oz prescription bottles(6). *Complement-fixing activity* was measured by modification of the Traub and Möhlmann procedure(7). As used in this laboratory,* the test is a direct semi-quantitative one, using guinea pig complement with overnight fixation at 4°C. The endpoint was read visually from 0 to +4 on 2-fold serially diluted samples. Readings from 0 to +2 were counted as belonging to the next lower dilution; readings of +3 or +4 were counted as belonging to the dilution used. Thus, a sample having a reading of +4 at a dilution of 1:256 and a reading of +1 at a

* Savan, M., Edward, A. G., Martinsen, J., Poppensiek, G. C., unpublished.

dilution of 1:512 was considered to have a CF titer of 256. *Protein* was determined spectrophotometrically by the ultra-violet adsorption method of Warburg(8), and also with Folin-Ciocalteau reagent(9), using crystalline bovine plasma albumin as a standard. *Source of complement-fixing antigen*: bovine tongue epithelium, infected with foot-and-mouth disease virus, type A, strain 119 (FMDV-A119), which had been stored in dry-ice chest for extended periods ranging up to 3 years, was homogenized in a Servall Omnimix as a 33% suspension in phosphate-buffered saline (0.02 M phosphate buffer, 0.10M sodium chloride), pH 7.5, in the presence of Genetron 113 (trifluoro trichloro ethane), and small amounts of toluene and octyl alcohol. The homogenate was stirred overnight at 4°C, and the aqueous extract was separated by centrifuging for 1 hour at 900 × g. The aqueous phase was then centrifuged in a Spinco Model L with a #30 rotor at 30,000 rpm for 2½ hours to sediment out infectious virus particles and other large particles. The supernatant fluid, whose virus titer was reduced by at least 5 log units, was centrifuged for 22-24 hours at 30,000 rpm. The supernatant liquid was discarded down to the level corresponding to the top of the sedimented pellet. The pellet plus this residual volume of liquid (about 3 ml) were resuspended in phosphate-buffered saline, and the clear aqueous CF-antigen concentrate was the starting material used in all experiments. One gram of tongue tissue yielded about 200 CF units (CF titer × ml). The CF titers of the aqueous concentrates ranged from 256 to 512 per ml, each ml containing about 50 mg of protein.

Results. Stability of the CF antigen was determined with respect to temperature, pH, enzymes and various methods of processing. Complement-fixing activity was completely retained for at least 3 months at 4°C, 1 week at room temperature (about 25°C), 4 hours at 37°C, and 30 minutes at 60°C. Activity was lost upon heating at 70°C. Adjustment of the CF-antigen solution to pH 5.0 for overnight at 4°C produced a precipitate having about 10% of the starting activity, with 50% of the starting activity remaining in the supernatant fluid. At pH 5.25, there was complete retention of activity, all in the supernatant fluid. Alkaline pH adjustment was accomplished by mixing the antigen solution with equal volumes of 4% sodium bicarbonate, 2% sodium carbonate, and 0.1N sodium hydroxide at final pH values of 8.3, 10.5, and 12.0, respectively. In the first 2 solutions CF activity was completely stable for at least 4 hours at room temperature, while there was a 75% loss of activity in the sodium hydroxide solution.

The effect of proteolytic and nucleolytic enzymes on the CF-antigen was determined. Trypsin and chymotrypsin (crystalline, Worthington) in concentrations as high as 5-10 mg per ml for 4 hours at 37°C had no effect on CF titer. Ribonuclease and deoxyribonuclease (crystalline, Worthington) acting for 24 hours at room temperature similarly produced no decrease in titer. The enzymes employed did not decrease significantly the total amount of proteins present in solution.

The CF-antigen concentrate was studied in the presence of the protein denaturant, urea, acting for 3 days at room temperature. A 2M urea solution did not affect CF activity, but with 6M urea, there was a loss of 75% of the starting activity. Trypsin did not have any effect on CF activity in these treated samples also.

Freeze drying of CF-antigen solutions in phosphate-buffered saline yielded powders retaining all the original activity. However, when precipitated preparations were dissolved in ammonium acetate solutions (no residue upon evaporation) and subsequently lyophilized, there were losses in activity ranging from 35 to 75%. The residual acidity of the acetic acid formed apparently was sufficient to cause this loss of activity.

Separation by precipitation of the CF-antigen was accomplished by various procedures. Exhaustive dialysis of CF-antigen concentrates against distilled water in the cold produced precipitates containing 75 to 90% of the total recovered activity which in turn ranged from 40 to 60% of the starting activity. The liquid within the dialysis membrane at the conclusion of the dialysis had a pH of 5.0 to 5.1 and this would account for the low

recovery. In order to avoid prolonged exposure of the CF-antigen to acid conditions, another deionization procedure involved shaking the CF-antigen solution with a prepared mixture of cation and anion exchange resins (Amberlite MB-3). However, this procedure also resulted in incomplete recovery of CF activity which was, in this case, partitioned fairly equally between precipitate and supernatant liquid. Isoelectric precipitation of the CF-antigen was studied over a pH range from 4.5 to 6.0 at intervals of 0.25 pH units. As indicated in the foregoing experiments on pH stability, there was a marked loss of activity at pH 5.0 and below. The precipitates formed contained very little to none of the remaining activity. Ammonium sulfate precipitations were made at pH 5.5 and pH 7.5 in the cold by adding that amount of a saturated solution of ammonium sulfate necessary to reach the desired percent of saturation in the CF-antigen solution. At both levels, activity was precipitated from 30 to 50% saturation, with less than 5% of the activity remaining in the supernatant fluid at the higher concentration of ammonium sulfate. However, recovery of activity from the precipitate at 50% saturation with ammonium sulfate was incomplete, sometimes being only half the starting activity. Paper electrophoresis of the re-dissolved precipitate showed, upon staining with bromphenol blue, a dark globulin area and a very faint albumin spot.

Ethanol fractionation was carried out at 2 different levels of ionic strength, the CF-antigen being either in a 0.01M phosphate buffer or in standard phosphate-buffered saline, with pH at 7.5 in both cases. Precipitations were done at 0°C with ethanol concentrations ranging from 10 to 40%. Recovery of CF-antigen was incomplete with activity spread between precipitates and supernatant fluids.

The CF-antigen was completely precipitated from aqueous solution by acetone in the cold, and the wet precipitate reconstituted in buffer with no loss of activity. Protamine sulfate and zinc ions precipitated part of the activity, while barium ions and sodium salts of ribonucleic acid and heparin had no effect.

Column chromatography, using several different adsorbents, was done at room temperature in 10 mm wide tubes, with column lengths ranging from 50 to 150 mm. The CF-antigen concentrate was loaded onto the column in a volume of 1-3 ml, with both charge and column having been equilibrated with the same buffer system. Elution was accomplished by stepwise increases in ionic strength of the eluting medium, at a flow rate of about 20 ml/hour. The eluate was collected in 4 ml fractions which were analyzed for protein content and CF-activity.

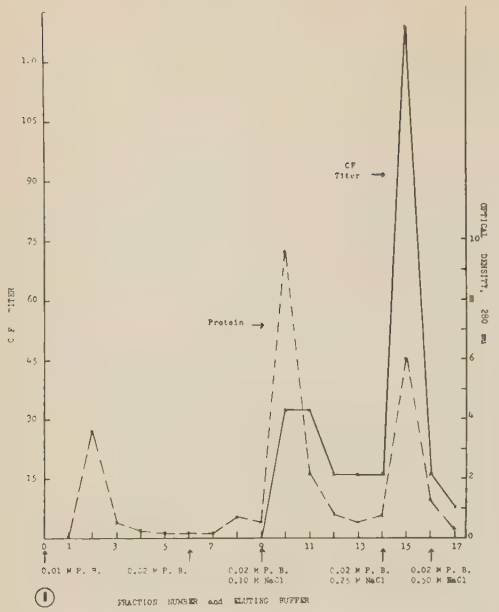
Results using a diethyl amino ethyl (DEAE) cellulose column buffered at low ionic strength are shown in Fig. 1. There was a 70% overall recovery of CF activity with a 40% recovery of proteins. The highest peak of activity contained 35% of the original charge and represented a 4-fold increase in purity. In another experiment, a DEAE cellulose column buffered at a higher ionic strength, 0.02M phosphate buffer containing 0.10M sodium chloride at pH 7.5, gave complete recovery of both CF activity and protein, with no significant purification.‡

Calcium phosphate columns prepared according to Tiselius(10), gave less than 1% recovery when equilibrated at low ionic strengths. Columns prepared in 0.02M phosphate buffer containing 0.10M NaCl yielded better recoveries of activity and fractions with purities comparable to those of the cellulose columns (Fig. 2).

The cation exchange resin, Amberlite CG-50, 400-600 mesh, equilibrated at pH levels ranging from 5.5 to 7.5 in isotonic buffers yielded unsatisfactory results, as did columns prepared of Dowex 50-x2, celite, and aluminum hydroxide (modified Willstatter Cv (11)).

Discussion. The small particle CF-antigen displays a markedly greater overall stability in comparison to the large particle having viral activity in the FMDV-A119 system. CF activity is retained after heating to 60°C for 30 minutes, while 90% of viral activity is lost after heating to 61°C for one-half minute

‡ While this paper was in preparation, Brown, F., and Cartwright, B., *Biochem. et Biophys. Acta,* 1959, v33, 343, reported the separation of crude suspensions of FMDV into two main fractions by chromatography on DEAE cellulose.



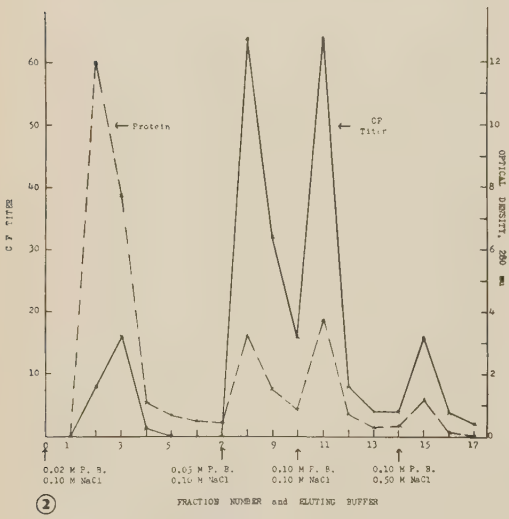


FIG. 1. Diethyl amino ethyl chromatography of complement-fixing antigen. Column and sample equilibrated with 0.01M phosphate buffer, pH 7.5. Column dimensions, 10 × 92 mm.

FIG. 2. Calcium phosphate chromatography of complement-fixing antigen. Column and sample equilibrated with 0.02M phosphate buffer containing 0.10M sodium chloride, pH 7.5. Column dimensions, 10 × 53 mm.

(12). The CF-antigen is stable over the pH range 5.25 to 10.5, while viral activity is most stable only at pH 7.0 to 7.5 and loses 90% of its activity in 14 hours at 4°C at pH 6.5 and 10.0. CF activity is resistant to the action of proteolytic enzymes and nucleases while viral activity in the tissue culture FMDV-A119 system, at least, is sensitive to the action of trypsin.† These differences in stability are of particular interest in view of the suggestion that the small particle can be derived by disintegration of the larger infectious unit(13).

Our data indicate that the CF-antigen should be classified as a euglobulin because it is insoluble in salt-free solutions. Also, it is precipitated by half-saturated ammonium sulfate and shows a predominantly globulin-type electrophoretic pattern.

The incompleteness of fractionation of CF activity by the various experimental technics may indicate the presence of more than one small particle containing CF activity. As determined by column chromatography, Figs. 1 and 2, the so-called small particle CF-antigen does have more than the one peak of activity. The presence of impurities and changes in eluting technic can modify this pattern. Therefore, it would also be of interest to determine the chromatographic distribution of CF activity in virus-free vesicular fluid from FMDV lesions.

Summary. The so-called small particle CF-antigen fraction, free of infectious virus particles and larger components, was prepared by centrifugation from bovine tongue epithelium infected with FMDV, type A, strain 119. The CF activity of this fraction was stable through the pH range of 5.25 to 10.5, withstood heating for 30 minutes at 60°C, and was resistant to the action of proteolytic and nucleolytic enzymes. It was precipitated by dialysis and by ammonium sulfate, ethanol, and acetone. Diethyl amino ethyl cellulose and calcium phosphate columns prepared at different ionic strengths were used in chromatographic purification. The small particle CF-antigen displays the properties of a euglobulin and is appreciably more stable than the large infective particle with which it is associated.

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Lipoprotein Lipase Metabolism in Experimentally Nephrotic Rats.* (25402)

RAY H. ROSENMAN AND SANFORD O. BYERS
(With technical assistance of Phyllis Nagel and Malcolm Smith)
Harold Brunn Inst., Mount Zion Hospital, San Francisco, Calif.

Our earlier studies(1,2) strongly suggested that deficiency of circulating albumin in experimentally nephrotic rats is in some manner responsible for abnormal intravascular retention of lipid and consequent hyperlipemia. The possibility of an abnormality of heparin or lipoprotein lipase metabolism as an associated causal factor in experimental nephrotic hyperlipemia also was suggested by earlier studies(3). Since it has been proposed(4,5) that in humans, nephrotic hyperlipemia results from a deficiency of lipoprotein lipase, the following studies were conducted.

Methods. Young, adult male rats of Long-Evans strain were used (average weight: 200 g). The nephrotic state was induced by injection of potent rabbit anti-rat kidney serum (6) 5 to 7 days prior to study and confirmed by demonstration of plasma albumin(7) content less than 1.2 g/100 ml and by presence of gross plasma lactescence and of marked hypercholesteremia(8). Disappearance of heparin from plasma of nephrotic rats was assayed, albeit crudely, by determining clotting time and by protamine titration of plasma obtained from groups of nephrotic and control rats bled at intervals following heparin injection. Each of a series of 10 nephrotic and 18 control rats was injected intravenously with heparin (0.5 mg/100 g). Five nephrotic and 12 control rats were bled with siliconized syringes, from the aorta 2 hours after heparin injection, and the remaining 5 nephrotic and 6 controls after 3 hours. Immediately after bleeding, 0.5 ml of each sample was placed in each of 3 serology tubes for clotting time determination and also in each of a 4-tube system containing serial dilutions of 0.01% protamine sulfate (prepared 24 hours previously). All tubes were kept in water bath at 37°C, and clotting times recorded as the time of average occurrence of a fast clot during gentle tilting at 30-second intervals. Lipoprotein lipase activity of nephrotic postheparin plasma was determined by ability to increase light transmission and to release unesterified fatty acids (UFA)(9) when added to a triglyceride substrate in normal rat plasma. For light transmission studies, plasma samples were obtained from 19 nephrotic rats, 10 minutes after injection of either 0.05 mg (5 rats) or 0.3 mg (14 rats) of heparin/100 g of body weight. Similar postheparin plasma samples were obtained from 16 normal, control rats. For further control purposes plasma

* Aided by Grants from N.I.H., P.H.S., Am. Heart Assn. and Western Nephrosis Soc.

samples were obtained from an additional 5 nephrotic and 7 normal rats which did not receive heparin. In each instance 0.2 ml of postheparin (or untreated) plasma was added to the test system, which consisted of 1.5 ml pooled normal rat plasma, 1 ml of 0.1 M phosphate buffer (pH 7.2) and 0.3 ml of 1:100 dilution of 50% coconut oil emulsion (Ediol, Schenley Labs.,) in 0.9% NaCl. The system was then incubated at 37°C in spectrophotometer cuvettes during 2-hour interval in which light transmission in the test system was measured at serial intervals in a Beckman model B spectrophotometer at 660 mμ. Distilled water was used as the blank. The results are expressed as percentage increases in light transmission which occurred during the experimental interval. For UFA studies, postheparin plasma samples were obtained as above from 22 nephrotic and 20 control rats after varying doses of heparin (0.02 to 1 mg/100 g body weight). For further control purposes, plasma of 4 untreated, normal rats also was obtained. UFA concentration of oil emulsion-normal rat plasma system described above was determined in each instance immediately and again 45 minutes after addition of 0.2 ml postheparin (or untreated) plasma, during which interval the samples were incubated at 37°C. All blood samples were obtained in chilled syringes from the aorta after a 30-hour fast, blood being collected in previously chilled tubes containing 1/10 volume of trisodium citrate, immediately separated by centrifugation in the cold for 15 minutes at 4,000 rpm and kept in an ice bath until use. No attempt was made to remove residual plasma lactescence from postheparin nephrotic rat plasma samples. To study rate of disappearance from plasma of lipoprotein lipase released in acute response to heparin injection, postheparin plasma samples were obtained 15, 45, 60 and 90 minutes after injection of heparin (0.4 mg/100 g) from 21 nephrotic and 19 control rats. These were then assayed by measuring the change in light transmission occurring in the test system described above, during a 60-minute interval following addition of postheparin plasma sample to be measured. Duration of lipolytic activity of postheparin nephrotic plasma also was studied in an additional series of 14 nephrotic and 15 control rats similarly injected with heparin and bled 10, 30, and 60 minutes later. In each instance the UFA content was determined immediately and 45 minutes after addition of postheparin plasma to the usual test system. To determine whether lipolytic activity would continue to be produced in plasma of nephrotic rats despite repeated heparin injection, 4 nephrotic and 4 control rats were each injected intravenously with 1 mg heparin at hourly intervals for 4 hours. One hour later each rat was injected with 0.1 mg heparin and postheparin plasma samples obtained 10 minutes later. In each instance ability of the postheparin plasma to increase light transmission and to release UFA in the same oil emulsion-normal rat plasma test system was determined as described above. The final experiment was concerned with determining ability of the same triglyceride substrate to be hydrolyzed by lipoprotein lipase when suspended in *nephrotic* rat plasma, the lipase being furnished by postheparin plasma obtained from normal rats. A series of 8 starved nephrotic rats was bled from the aorta using cold syringes, and the plasma immediately separated in a refrigerated centrifuge. All visible plasma lactescence was then removed by centrifuging at 4°C for 30 minutes at 28,000 rpm and the cleared subnatant plasma kept in ice bath until used. Each plasma sample was divided into paired aliquots of 3 ml and to each aliquot was added 0.35 ml of the 1:100 dilution of coconut oil emulsion and 0.25 ml of postheparin plasma, obtained 10 minutes after injection of a normal 30-hour-starved rat with 1 mg heparin. In addition, 60 mg of powdered bovine serum albumin (Armour Fraction V) was added to one of each paired aliquots. All samples were incubated at 37°C for 2 hours, during which light transmission was measured at intervals. UFA also was determined immediately and 45 minutes after addition of postheparin plasma. For control purposes, similar studies were done using plasma obtained from 5 starved, normal rats.

Results. 1. *Effect of heparin on blood clot-*

TABLE I. Effect of Intravenous Heparin on Clotting Time of Nephrotic Rats.

Type of rat	No. of rats	Avg wt, g	Total cholesterol, mg/100 ml	Clotting time, min.	Avg protamine-induced clotting time, min. .001 mg	.002 mg	.004 mg	.008 mg
*Rats bled 2 hr after heparin**								
Nephrotic	5	230	366 (159-502)†	20.5 (15-27)	17.0 (14.0-22.5)	14.7 (11.0-19.0)	11.0 (8.0-14.5)	7.0 (5.0-8.0)
Control	12	245	54 (41-69)	22.9 (10-35)	19.0 (12.5-23.0)	10.5 (7.3-16.5)	5.2 (4.0-6.3)	4.8 (3.5-6.3)
*Rats bled 3 hr after heparin**								
Nephrotic	5	210	417 (244-703)	17.2 (13-22)	14.5 (10.0-17.0)	10.1 (9.5-11.0)	6.5 (4.5-8.5)	5.2 (4.3-8.0)
Control	6	225	61 (42-74)	11.0 (7-17)	11.2 (8.3-21.0)	6.5 (5.0-8.3)	5.5 (4.0-6.5)	5.0 (3.3-6.5)

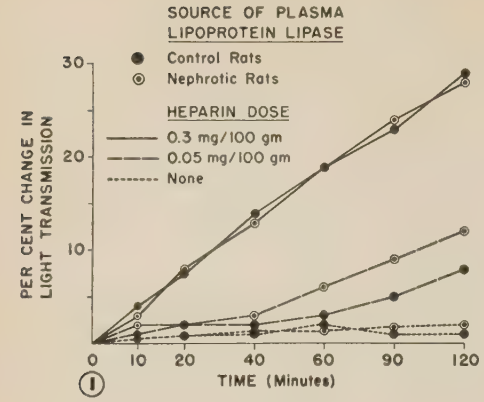
* 0.5 mg/100 g. † Range of values.

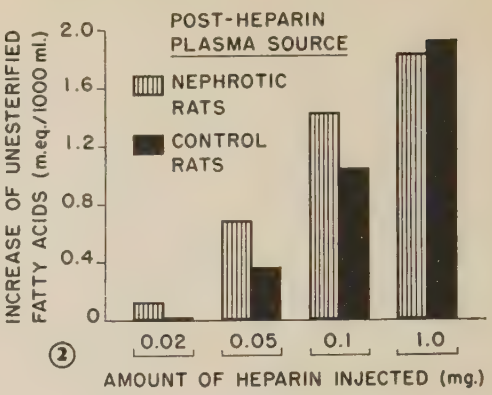
ting time of nephrotic rats. Table I presents results of blood clotting time of postheparin nephrotic and control rat blood. Blood clotting times of nephrotic rats averaged 20.5 minutes in those bled 2 hours, and 17.2 minutes in those bled 3 hours, after heparin injection, compared to an average of 22.9 minutes and 11 minutes in their respective controls. Table I also shows amount of protamine sulfate required to neutralize heparin-induced prolongation of clotting time of nephrotic rats' plasma at each sampling interval, was not less than that required for comparable effect in their respective control rats' plasma.

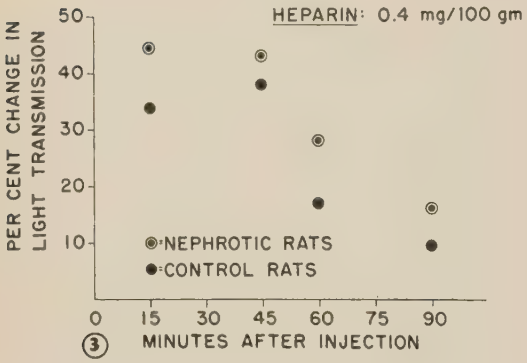
2. *Ability of nephrotic rat to release lipoprotein lipase following heparin administration.* The first experiment was concerned with ascertaining nephrotic rat's ability to release lipoprotein lipase into its plasma, immediately following heparin administration. Fig. 1 shows percentile average increase of light transmission in coconut oil emulsion-normal rat plasma test system, following addition of plasma obtained from groups of nephrotic and control rats. Whereas no change in light transmission was noted following addition either of preheparin nephrotic or of control rats' plasma, a progressive increase of light transmission was consistently observed following addition of postheparin nephrotic plasma, of considerably greater magnitude in instances where the latter was obtained from nephrotic rats injected with larger quantity of heparin. At both dosage levels, rate and magnitude of increase of light transmission in the test system following addition of postheparin nephrotic rats' plasma were similar to changes induced by addition of postheparin plasma obtained from their respective controls. The plasma total cholesterols of nephrotic rats averaged 255 mg/100 ml (range: 152-527).

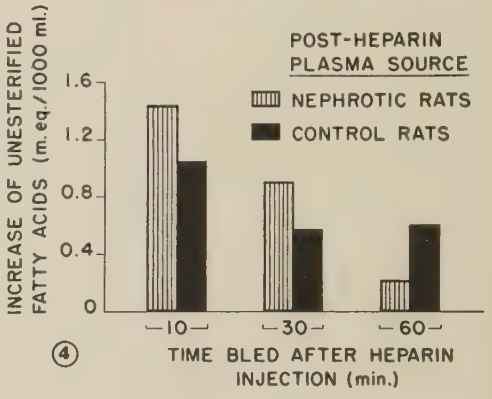
Fig. 2 shows the increase of UFA in the same oil emulsion-normal rat plasma test system which occurred during 45-minute interval following addition of plasma obtained from nephrotic and control rats variously injected with heparin (0.02 to 1 mg/100 g weight). In each instance the average increase of UFA effected by postheparin nephrotic rats' plasma was not less than that induced by their respective control rats' postheparin plasma. As expected, in both groups the magnitude of UFA increase was progressively greater when postheparin plasma was obtained from rats given the larger dosage of heparin. On the other hand, no increase of UFA was noted in the test system following addition of plasma obtained from control rats not injected with heparin, the initial UFA content in these rats averaging 0.540 meq/l (range: 0.432-0.592) and 45 minutes later averaging 0.502 meq/l (range: 0.412-0.540). Plasma total cholesterol of nephrotic rats averaged 357 mg/100 ml (range: 163-457).

The second experiment compared *duration* of lipolytic activity of postheparin nephrotic and normal rat plasma. Fig. 3 shows percentage average increase of light transmission occurring in oil emulsion-normal rat plasma test system during 60-minutes following addition of test postheparin nephrotic and control rats' plasma, variously obtained at 15 to 90









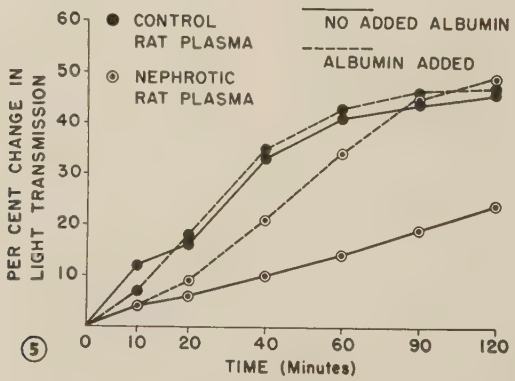


FIG. 1. Lipemia-clearing activity of post-heparin nephrotic rats' plasma.
FIG. 2. Lipolytic activity of post-heparin nephrotic rat's plasma.
FIG. 3. Lipemia-clearing activity of nephrotic rat's plasma at intervals after intrav. heparin.
FIG. 4. Lipolytic activity of nephrotic rat's plasma at intervals after intrav. heparin.
FIG. 5. Effect of albumin on lipemia-clearing of triglyceride in nephrotic rats' plasma.

minutes following heparin injection. At each interval, the lipolytic activity of postheparin nephrotic rats' plasma, thus assayed, was not less than that of control rats. Fig. 4 shows the average increase in UFA content induced in the same oil emulsion-normal rat plasma system by addition of postheparin plasma obtained from nephrotic and control rats again bled at various intervals following heparin administration. The postheparin plasma obtained from nephrotic rats bled 10 and 30 minutes after heparin injection effected com-

parable or slightly gerater increases of UFA than did their respective control rats' postheparin plasma, the converse being found among rats bled 60 minutes after heparin injection. However, the number of rats involved in this experiment was relatively small, and the observed differences are not significant. The plasma total cholesterols of nephrotic rats averaged 298 mg/100 mg (range: 163-457).

The third experiment compared lipolytic activity of plasma obtained from nephrotic and control rats after repeated injections of heparin. Postheparin plasma thus obtained from nephrotic rats increased light transmission an average of 28% (range: 21-34) during 60-minute interval following addition to the oil emulsion-normal rat plasma test system, and increased the UFA content of the test system from initial average of 0.485 meq/l (range: 0.304-0.577) to 1.949 meq/l (range: 1.533-3.010) 45 minutes later. Addition of control rats' postheparin plasma to the test system similarly increased light transmission an average of 27% (range: 22-30) and increased the UFA content from an initial average of 0.347 meq/l (range: 0.149-0.600) to an average of 1.662 meq/l (range: 1.209-2.124).

3. *Ability of nephrotic plasma to support lipolysis of a triglyceride substrate.* The final experiment was concerned with studying ability of normal rat postheparin plasma to effect lipolysis of the same triglyceride substrate used above when suspended in nephrotic plasma ultracentrifugally "cleared" of its own excess triglyceride content. Addition of normal rat postheparin plasma to the oil emulsion comparably increased the UFA content whether the triglyceride substrate was suspended in normal or nephrotic rat plasma, although the albumin content of the normal rat plasma averaged 2.74 g/100 ml (range: 2.01-2.51) compared to an average of 0.79 g/100 ml (range: 0.62-1.15) in the nephrotic plasma. Thus the UFA content increased an average of 1.305 meq/l (range: 0.517-2.074) in the oil-nephrotic rat plasma and 1.283 meq/l, range: 1.034-1.727) in the oil-normal rat plasma following addition of the normal rat postheparin plasma. These values were not significantly altered when albumin was added to the same test system, averaging 1.682 meq/l (range: 1.271-2.283) and 1.076 meq/l (range: 0.449-1.423), respectively, under these circumstances.

During the 2-hour experimental interval following addition of normal rat postheparin plasma, light transmission increased an average of 46% in the control, oil emulsion-*normal* rat plasma system, and an average of 47% in the paired aliquots also containing added albumin (Fig. 5). In contrast, light transmission increased an average of 24% during the same interval in the oil emulsion-nephrotic rat plasma test system following addition of the same postheparin plasma, and an average of 48% in paired aliquots containing added albumin.

Discussion. Since lipoprotein lipase production is not related to the anticoagulant action of heparin, the results of the first experiment suggest that the experimental nephrotic state in rats is not associated with any abnormality in plasma disappearance of exogenously administered heparin.† Moreover, the data can do no more than imply a similar normality in nephrotic rats' handling of endogenously derived heparin.

It is believed significant, however, that our studies failed to detect any abnormality in lipoprotein lipase response of the nephrotic rat to administered heparin. Thus, whether plasma was obtained from the nephrotic rat immediately, somewhat later, or even after repeated doses of heparin, it exhibited as much lipase activity as did plasma of rats similarly injected with heparin.

That nephrotic rat postheparin plasma effected release of UFA and optical "clearing" of the triglyceride substrate comparable to that induced by normal rat postheparin plasma also fails to support the contention that such experimental nephrotic rat plasma contains an inhibitor of lipoprotein lipase (12). The presence of such an inhibitor has

† Although the data actually suggest a delayed rate of heparin removal in the nephrotic rat, any statistical evaluation of such a conclusion did not appear warranted in view of the relative crudeness of the assay method employed.

not been ruled out, however, since in the human at least, it may be contained in the fatty layer of ultracentrifuged, hyperlipemic plasma (13), removed from the nephrotic plasma used in final experiment.

In the final experiment no abnormality was detected in ability of nephrotic plasma to support lipase-induced release of UFA from a triglyceride substrate suspended in such plasma. The fact that concomitant optical "clearing" of this triglyceride substrate proceeded at a slower rate and was of lesser magnitude when suspended in nephrotic plasma compared to normal rat plasma is not surprising since lipase-induced release of UFA is only poorly related to optical density changes and occurs even in absence of optical "clearing"(14). Addition of extra albumin to the hypoalbuminemic, nephrotic rat's plasma appeared to normalize the ability of such plasma to support continued lipase-induced "clearing" of the added triglyceride substrate. This would appear only to confirm the well-known role of albumin as an acceptor for UFA(10,11) and the fact that continued optical "clearing" of a triglyceride substrate by lipoprotein lipase is prevented by release of UFA in excess of that which can be bound by available albumin(10,11,14).

Although adequate albumin appears essential for sustained lipolysis *in vitro*(10,11,14) and for sustained lipase-induced, lipemia-clearing *in vivo* in the nephrotic rat(3), its known role as an acceptor of UFA(10,11) does not actually explain the mechanism by which severe renal loss of albumin and hypoalbuminemia appear sequentially to induce hypertriglyceridemia(1,2), hyperphospholipemia and hypercholesteremia(15). Thus our exeriments do not indicate any actual inability of lipase to release UFA in nephrotic plasma, and indeed, intravascular lipolysis of triglyceride may be neither a prerequisite nor a significant physiological mechanism for plasma egress of its fatty acid moiety(16,17).

Summary. Heparin-activated lipolytic mechanisms were studied *in vitro* in rats with nephrosis experimentally induced by injection of antikidney serum. The nephrotic rat was found normally capable of releasing lipoprotein lipase following heparin administration. The nephrotic rat's plasma also was found normally capable of supporting lipoprotein lipase-induced hydrolysis of a triglyceride substrate.

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Effect of Inosine on Iron Absorption in Rats.* (25403)

Barbara Cheney and Clement A. Finch
Dept. of Medicine, University of Washington School of Medicine, and King County Central Blood Bank, Seattle, Wash.

In a series of studies Mazur and his collaborators(1) presented data to indicate that iron may be mobilized from tissue cells through reduction and liberation of ferritin iron by xanthine oxidase. This mechanism may be activated through initial reduction of xanthine oxidase by injected nucleosides. Mazur's studies deal chiefly with mobilization of iron from existent tissue stores, and the effect of injected inosine on transport of iron across intestinal mucosa is examined.

Materials and methods. Iron absorption as affected by inosine, was studied in male Sprague-Dawley rats weighing 200 to 300 g. All animals were given 5 mg of iron intramuscularly (Imferon†) 10 to 14 days before experiments to eliminate the possible influence of iron deficiency on our results. Approximately 10 μc of Fe^{59} as $FeCl^3$ in volume of 0.5 ml of acidified saline was administered by intubation to the etherized rat after 24 hour fast. Inosine‡ was dissolved in saline and injected intravenously unless otherwise stated. Animals were sacrificed by exsanguination 8 days later while under ether anesthesia. The liver was perfused free of blood. Total blood activity was determined from aliquot of blood removed and an assumed blood volume of 50 ml/kg. Tissue counting was performed in most instances by placing the tissue or its aliquot directly in a vial for counting. Carcass analysis was performed by digesting the entire autoclaved carcass in various fractions in nitric acid and analyzing an aliquot from each fraction. A scintillation counter was employed for counting and a total of at least 4,000 counts compiled on each sample. Samples were usually in counting range of over 20 times background, *i.e.*, 2,000 to 20,000 counts/minute.

Results. Absorption studies are summarized in Table I. When given intravenously, inosine had a consistent effect in increasing amount of iron absorbed. This effect was maximal when inosine and iron were administered simultaneously. Inosine was without effect if given orally. The effect of inosine injection on iron absorption, was present over a range of iron dosage (0.07 to 4.6 mg/kg). The amount of inosine required for the effect appeared to be as little as 0.03 g/kg.

Body distribution of absorbed radioiron with and without parenteral administration of inosine is shown in Table II. Liver and blood contained some 94% of total carcass activity in controls and 90% in inosine-treated animals. Our studies indicate that inosine given at time of intravenous injection of transferrin-bound iron also produces a greater deposition of iron in the carcass exclusive of blood, marrow and liver.

Discussion. Any information which provides an understanding of the intracellular handling of iron would appear important to our understanding of iron metabolism. Mazur's studies(1) or the effect of xanthine oxidase *in vivo* in releasing iron from the ferritin reservoir of liver provide a possible insight into this problem. We have been impressed with the similar and the synchronous behavior of various body cells in response to certain situations which alter iron kinetics. Thus following bleeding, the reticulo-endothelial cell, the gastrointestinal-mucosal cell and the placental cell all show an increase in their release of iron(2,3). The results here reported indicate that inosine influences iron transport across the intestinal mucosa as well as in the liver. The magnitude of this effect however was small (50 to 100% above normal absorption) and suggests that the much greater increase observed in iron depletion or in idiopathic hemochromatosis (300 to 600%) may

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† Kindly supplied by Lakeside Labs, Milwaukee, Wisc.

‡ Obtained from Schwarz Labs, Mount Vernon, N. Y. and Nutritional Biochemicals Corp., Cleveland, O.

TABLE I. Absorption Studies in Rats.*

Exp.	Condition	No. of animals	Avg wt, g	Amount Iron, mg/kg	Amount Inosine, g/kg	% uptake Blood	% uptake Liver
1 A	Controls	9	313	2.56	(Saline)	2.0 ± .73†	.30 ± .14†
B	Inosine 0°	3	323	2.78	.11	3.5 ± 1.08	.77 ± .13
C	4° before 0 time	4	312	2.56	.10	1.4 ± .39	.30 ± .05
D	2° " "	4	321	2.49	.11	2.2 ± 1.20	.38 ± .12
E	2° after 0 time	4	317	2.52	.10	2.5 ± 1.32	.63 ± .48
F	4° " "	4	339	2.36	.10	2.5 ± .93	.41 ± .19
2 A	Controls	10	347	2.31		.91 ± .24	.16 ± .02
B	Inosine 0 time	5	315	2.54	.29	1.16 ± .69	.39 ± .31
C	*Idem*	5	327	2.45	.18	1.30 ± .16	.32 ± .06
D	"	5	342	2.34	.09	1.45 ± .69	.39 ± .06
E	"	5	311	2.57	.03	1.13 ± .49	.30 ± .14
3 A	Controls	4	340	.07	.09	4.61 ± 2.69	.89 ± .46
	Inosine 0 time	4	320	.08	.09	7.84 ± 5.81	1.13 ± .75
B	Controls	4	345	.29	.09	1.09 ± .51	.23 ± .04
	Inosine 0 time	4	328	.30	.09	2.73 ± .76	.43 ± .17
C	Controls	4	332	1.51	.09	1.10 ± 1.48	.23 ± .19
	Inosine 0 time	4	332	1.51	.09	1.58 ± .36	.28 ± .09
D	Controls	3	326	4.60	.09	.89 ± .22	.14 ± .06
	Inosine 0 time	3	327	4.59	.09	.92 ± .44	.37 ± .37
4 A	Controls	8	275	.36		2.56 ± .80	.54 ± .12
B	Inosine P.O.	5	277	.36	.11	2.27 ± .58	.52 ± .09
C	*Idem*	6	318	.31	.19	2.40 ± .67	.64 ± .18

† ± stand. dev. Besel's correction, $S = \sqrt{\frac{\Sigma x^2}{n} \times \frac{n}{n-1}}$. x = individual results

* Animals were sacrificed 8 days after administration of radioiron.

be mediated in another manner. These data do not exclude a non-specific effect of inosine, for example, on intestinal blood flow. They merely define the positive effect of inosine on iron absorption and its magnitude. Observations on distribution of absorbed radioiron in the carcass of rats, indicate that the difference in absorption is a true difference and not dependent on a change in internal distribution of iron. They also indicate that blood and liver analyses suffice in measurements of absorption of iron in non-pregnant animals.

TABLE II. Distribution of Absorbed Iron at 8 Days.*

	Control group	Inosine*
No. of animals	6	6
Wt	337 (308–360)	338 (296–370)
	% of admin. iron	
Blood activity	4.34	4.93
Liver "	1.23	1.35
Carcass "	.36	.66
Total "	5.93	6.93

* Dose of 0.3 mg/kg of iron was given by mouth and at same time 30 mg of inosine was given intrav. Liver was not perfused.

Summary. Inosine administered intravenously at time of iron absorption increased the amount of iron absorbed by 50 to 100% in rats.

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Hypocholesteremic Effect in Man of Benzmalecene: An Inhibitor of Cholesterol Biosynthesis. (25404)

Stanley S. Bergen, Jr., Theodore B. Van Itallie and W. H. Sebrell
Dept. of Medicine, St. Luke's Hospital, and Inst. of Nutrition Sciences, Columbia University, N. Y. City

Benzmalecene, N-(1-methyl-2,3-di-p-chlorophenylpropyl)-meleamic acid (α isomer), has been shown by Huff and Gilfillan(1) to inhibit *in vitro* incorporation of mevalonic acid into cholesterol. These investigators also have demonstrated that benzmalecene lowers serum cholesterol when administered to rats(1). To determine the effect of benzmalecene in human subjects, it was administered to a group of patients for periods varying from one to 7 weeks.

Procedure. Nine patients were given 500-1000 mg of benzmalecene (free acid or the sodium salt)* per day for 10 to 53 days. Serum cholesterol levels were determined and measurements of hepatic, renal and hematopoietic function were made before, during and after benzmalecene treatment. Temperature, blood pressure, pulse, weight, appetite and urinary output were closely followed. Patients were studied in the hospital and were on the regular hospital diet, except when salt restriction was necessary. No other medications or dietary supplements known to have a hypocholesteremic effect were administered during study. All 9 patients had coronary artery disease; in addition, one had primary bronchogenic carcinoma, and one diabetes mellitus. Six had elevated serum cholesterol levels. Ages of the patients ranged from 37 to 81 years. Benzmalecene was administered either as the sodium salt or the free acid in amounts of 250 or 500 mg twice a day, after breakfast and in the afternoon. Two or more serum total cholesterol and cholesterol ester determinations were made before starting benzmalecene. Thereafter, determinations were made 2 or 3 times a week while patients were receiving benzmalecene, and at periodic intervals after cessation of treatment. Tests of renal, hepatic and hematopoietic function were performed once a week. Serum total cholesterol and cholesterol ester levels were done by the Schoenheimer-Sperry method(2).

Results. The effect of benzmalecene on serum cholesterol was evaluated in 6 of the 9 subjects studied. One patient began to show a lowering of serum cholesterol while on benzmalecene but left the hospital after only 8 days of treatment. Two patients developed laboratory evidence of possible toxic side-effects of benzmalecene and its effect on serum cholesterol was therefore obscured. In 6 patients serum total cholesterol levels were lowered 11.4 to 25.3%, the average being 18 $\pm$ 4.67% (Table I). This drop was statistically significant at the 0.01 level. The 5 patients receiving either 1000 mg of the benzmalecene free acid or 500-1000 mg of the sodium salt per day had decreases of 15% or more. Depression of serum cholesterol level occurred within a week after the drug was begun; after discontinuance of benzmalecene it took approximately a week for serum cholesterol to return to control values. None of the patients who received the benzmalecene had any complaints attributable to the drug. Appetite and body weight were not affected. The 2 patients who developed laboratory evidence of possible toxicity had ester decreases to a greater degree than total cholesterol and an eosinophilia of 12% and 16% respectively. One patient became slightly icteric and complained of generalized pruritis. He was receiving chlorpromazine concurrently and withdrawal of both medications was followed by clearing of jaundice and disappearance of pruritis. These manifestations may have been due to chlorpromazine since they did not recur with a subsequent 4 day "challenge" with benzmalecene. The other patient was on no other medication at time of study and sodium sulfobromophthalein (BSP) retention at 45 minutes increased from 8% to 21% during benzmalecene treatment. Three

* Supplied by Merck Sharp & Dohme Research Lab., Rahway, N. J.

TABLE I. Changes in Serum Total Cholesterol during Administration of Benzmalecene.

Age (yr) and sex of patients		Medication	Dose/day	Avg control* serum total cholesterol, mg % (range)	Avg % decrease	Range of levels during treatment, mg %
72	♂	Sodium benzmalecene	1000	278 (274–282)	19 4	257–208
81	♀	Benzmalecene (free acid)	750	200 (198–202)	11.4	202–164
58	♂	*Idem*	1000	299 (293–308)	17.0	256–240
68	♀	"	"	318 (304–332)	25.3	245–229
53	♀	"	"			
		Sodium benzmalecene	500–1000	274 (255–292)	15.5	283–189
66	♀	*Idem*	500	282 (275–290)	19.8	256–202

* Two or more successive determinations.

other patients had depressions of serum alkaline phosphatase levels from control values of 7-10 to 1.5-3.0 King-Armstrong units.

Discussion. Benzmalecene appears to be an effective hypocholesteremic agent in man. There was no evidence of escape from the effect of the medication in these studies, but there seemed to be a definite minimal dose level below which an appreciable hypocholesteremic response could not be obtained. Two of the benzmalecene treated patients (one on chlorpromazine concurrently) developed evidence of possible liver toxicity and eosinophilia. Blondheim(3) has reported probenecid inhibition of BSP excretion in normal human subjects receiving probenecid. The similar pharmacologic action of benzmalecene and probenecid may well explain this effect of benzmalecene. However, the excessive reduction in esterified cholesterol and the eosinophilia occurring in 2 of the patients under study seem best explained as possible toxic manifestations of the drug. Peck has noted elevation of alkaline phosphatase levels and increased BSP retention in dogs given benzmalecene orally in relatively high doses. In these animals the alkaline phosphatase elevations returned to control levels on continued therapy with the same dose (personal communication).

Summary. Benzmalecene in 0.5-1.0 g/day of the free acid (or sodium salt) was studied in 6 patients for 10 to 53 days. Serum total cholesterol levels in this group were decreased by an average of 18%. Accordingly, this drug shows promise as serum cholesterol depressant. However, treatment with benzmalecene was associated with decreases in serum alkaline phosphatase and a drop in cholesterol ester levels in disproportion to decrease of serum total cholesterol. These changes as well as occurrence of eosinophilia in 2 subjects suggest that benzmalecene in amounts used in this study may prove to be too toxic for clinical use.

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Benzmalecene: Inhibition of Cholesterol Biosynthesis and Hypocholesteremic Effect in Rats. (25405)

JESSE W. HUFF AND JAMES L. GILFILLAN (Introduced by H. J. Robinson)
Merck Inst. for Therapeutic Research, Rahway, N. J.

An agent capable of inhibiting biosynthesis of cholesterol by mammalian systems, would be of possible clinical interest in various hypercholesteremic states. The discovery that 2-C^{14}-mevalonic acid(1) is utilized by rat liver preparations for biosynthesis of cholesterol with an efficiency far greater than any previously tried precursor has made possible the study of a number of compounds which would inhibit this system. This paper describes results obtained with benzmalecene,* a potent inhibitor arising from these investigations.

Methods and materials. In vitro experiments. A cell-free rat liver homogenate system which synthesizes cholesterol was prepared employing the technic described by Bucher(2), as modified by Rabinowitz and Gurin(3). Livers were obtained from male albino rats† received at 75-80 g weight and maintained for one week on Purina Laboratory Chow. The substrate employed was 2-C^{14}-DL-mevalonic acid (2-C^{14}-MVA) having a specific activity of 0.0125 mc/mM as measured by liquid scintillation counting. Each tube‡ contained 0.4 mg of 2-C^{14}-MVA, 1 ml of rat liver homogenate and 0.2 mg each of adenosine triphosphate (ATP) and diphosphopyridine nucleotide (DPN). The volume was made to 2.2 ml with pH 6.9 buffer which has been previously described(3). The gas phase was 95% O_2 and 5% CO_2. Incubation was carried out at 37° for 4.5 hours with agitation. Two mg of carrier cholesterol were added after incubation and the cholesterol was isolated and counted as the digitonide according to a previously described procedure(3). The benzmalecene was solubilized with an equivalent amount of NaOH in small amount of water and diluted to concentration of 1 mg/ml with the pH 6.9 buffer solution. Aliquots of the solubilized compound were added to the incubation mixture described, at the expense of the buffer solution. *In vivo experiments.* Holtzman male albino rats were obtained at 130-140 g in weight and maintained on Purina Laboratory Chow for one week. The rats were then randomized according to weight into several groups of 20 rats each and placed on Purina Lab Chow diet to which 10% lard had been added. Benzmalecene was administered by incorporation in the diet. After 10 days, 2 ml blood samples were taken by cardiac puncture under light Nembutal anesthesia and placed in tubes containing 0.2 ml of 0.4 M sodium citrate. The plasma was analyzed for total cholesterol by the method of Abell *et al.*(4).

Results. In vitro experiments. Table I shows results typical of those obtained when benzmalecene is added in increasing concentrations to a rat liver homogenate system which converts mevalonic acid to cholesterol. As the concentration of benzmalecene is increased there is a sharp depression in cholesterol synthesis with maximum inhibition being obtained at the 0.15 mg/tube level or about 1.9×10^{-4} M.

To determine if the inhibition by benzmalecene of this multi-enzyme system was competi-

TABLE I. Inhibition of *In Vitro* Incorporation of 2-C^{14}-Mevalonic Acid into Cholesterol by Rat Liver Homogenates.

Compound added*	Level, mg/tube	Recovered cholesterol, cpm/mg C	% inhibition
None		1700	
Benzmalecene	.05	975	43
	.075	852	50
	.10	320	81
	.15	9	99
	.20	2	100

* Each tube contained 400 μg of 2-C^{14}-dl-mevalonic acid (specific activity = .0125 mc/mM).

* Generic name for N-(1-methyl-2,3-di-p-chlorophenylpropyl)-maleamic acid (α-isomer). This compound was supplied by Dr. E. M. Schultz of Merck Sharp & Dohme Research Labs., West Point, Pa.

† Sprague-Dawley strain obtained from C & L Breeders, East Greenville, Pa.

‡ Screw cap culture tubes (20 x 125 mm).

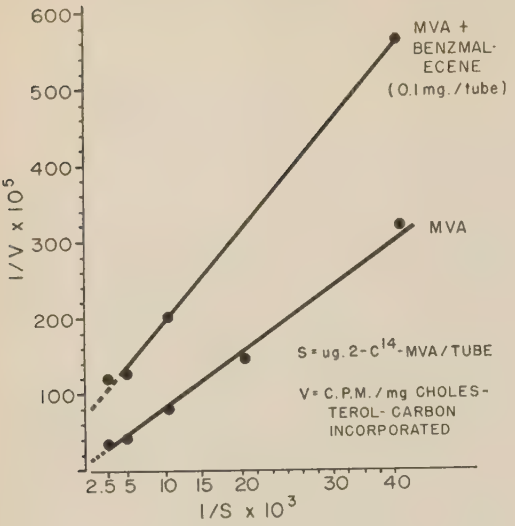

FIG. 1.

tive or non-competitive, the Lineweaver-Burk (5) method of plotting was employed. The incorporation of mevalonic acid into cholesterol over a range of 25 to 400 μg per tube was determined in the absence of inhibitor and then in the presence of a single level of benzmalecene in all tubes. The data, when plotted according to Lineweaver and Burk(5) (Fig. 1), indicate the over-all inhibition to be non-competitive. *In vivo experiments.* Table II illustrates the effect of benzmalecene on plasma cholesterol level of rats when the compound was administered in the diet for 10 days as the free acid and as the sodium and calcium salts. The free acid at levels of 0.1, 0.2 and 0.4% in the diet reduced plasma cholesterol level by 12, 20 and 26% respectively. Little or no effect on body weight gain was apparent. Due to the possibility of poor absorption of the free acid form, the sodium salt was also studied. This salt was more effective, producing decreases in plasma cholesterol of 22, 29 and 39% at levels equivalent to those employed for the free acid. The hygroscopic nature of the sodium salt prompted the testing of the calcium salt. This material, although appearing somewhat less active than the sodium salt at the 2 lower levels, was equal in activity at the 0.4% level. The 2 salts caused some depressing effect on body weight gain.

Discussion. The results of the *in vitro* experiment demonstrate that benzmalecene is a potent metabolic inhibitor of cholesterol synthesis. The exact point at which the metabolic block is exerted in the pathway of mevalonic acid to cholesterol is not known.

When benzmalecene, which blocks the synthesis of cholesterol by rat liver *in vitro,* was incorporated into a chow diet containing lard, a significant reduction in plasma cholesterol level resulted. Benzmalecene has been shown in other studies from these laboratories to produce a prompt depression in blood cholesterol in the dog when given orally at 50 mg/kg/day(6). The inhibition of *in vitro* cholesterol synthesis by benzmalecene, coupled with its effect on plasma cholesterol level in *in vivo* experiments, suggests, but does not necessarily prove that the latter effect is a direct result of an *in vivo* reduction of cholesterol synthesis.

A number of other substances have been re-

TABLE II. Effect of Benzmalecene and Its Sodium and Calcium Salts on Plasma Cholesterol Level of Rats.

	Benzmalecene (free acid)		Benzmalecene (sodium salt)		Benzmalecene (calcium salt)	
% compound in diet	Plasma cholesterol, mg %	Wt gain, g	Plasma cholesterol, mg %	Wt gain, g	Plasma cholesterol, mg %	Wt gain, g
0	64.0	53	60.1	53	62.7	46
.1	56.2	50	46.8	44	51.0	47
.2	51.3	52	42.9	37	45.3	39
.4	47.5	51	36.4	31	36.8	
L.S.D.*$_{.05}$	2.9 mg		3.2 mg		3.8 mg	
" *$_{.01}$	3.8		4.3		5.1	

$$* \text{L.S.D.} = t \times \gamma \times \sqrt{\frac{1}{N_1} + \frac{1}{N_2}}.$$

ported which inhibit hepatic synthesis of cholesterol and lower blood cholesterol; α-phenylbutyric acid(7,8), α-p-bi-phenylbutyric acid (9,10) and vanadium salts(11,12).

It is not known if an increased hepatic synthesis of cholesterol is a contributing factor in the etiology of the various abnormal metabolic states in which cholesterol has been implicated. The clinical study of benzmalecene in these conditions should be useful in obtaining information on their etiology and may offer a practical method of combating hypercholesteremic states.

Summary. Benzmalecene inhibited *in vitro* incorporation of 2-C^{14}-mevalonic acid into cholesterol by rat liver homogenates. The inhibition proved to be non-competitive. Oral administration of this compound to normal rats resulted in a significant reduction in plasma cholesterol.

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Erythropoietin. I. Analysis of Radioiron Assay in Fasted Rats, Using Cobalt as a Reference Standard.* (25406)

R. J. SCHLUETER, H. NORGELLO AND W. F. WHITE
(Introduced by R. E. Thompson)
Central Research Labs., Armour and Co., Chicago, Ill.

In the past few years research on erythropoietin has expanded rapidly and the number of papers in this field has greatly increased. Unfortunately, however, it has been difficult to compare assay results between laboratories because there has been no reference standard. Gordon(1) documented the need for such a material. We attempted to remedy the situation by choosing cobalt† as a readily available primary reference standard in the radioiron assay developed by Fried *et al.*(2). Using the starved rat as subject, 5 μmoles of cobalt gives a substantial response above the saline blank and therefore has been designated as the one unit response. Further studies have been made in the 1 to 2 unit range, comparing cobalt to various erythropoietin fractions. Finally, a partially purified fraction from anemic sheep plasma has been carefully standardized against cobalt and has been adopted by our laboratory as a day-to-day working standard.

Methods. Normal, 2 month old, male Sprague-Dawley rats, weighing 175 to 200 g were used. The rats were acclimated to the animal room for 4 days, and maintained on Rockland R-4 rat diet. At start of assay all food was withdrawn, but water was supplied *ad lib.* Samples were prepared in 0.05 *M* $NaPO_4$, 0.15 *M* NaCl, pH 6.8 (saline). After 30 hours fasting, the first 2 ml sample was injected intravenously (tail vein) under light

* This paper is based on work done under Contract with Atomic Energy Comm.

† Here the simple term "cobalt" is understood to mean "cobaltous ion". Reagent grade cobalt chloride ($CoCl_2 \cdot 6H_2O$) has been used as source of cobaltous ion.

TABLE I. Analysis of Effect of Cobalt on Fe^{59} Uptake in Fasted Rats in 7 Assays. Consecutive experiments in which both levels of cobalt were assayed.

Saline		5 μM Co^{++} dose		10 μM Co^{++} dose		Slope	
No. rats	% uptake Fe^{59}	No. rats	% uptake Fe^{59}	No. rats	% uptake Fe^{59}	b*	Lambda
6	2.86 ± .45	6	8.71 ± 1.70	6	12.93 ± 5.02	14.02	.268
6	4.41 ± .91	6	6.31 ± 1.07	6	9.46 ± 1.64	10.45	.132
6	3.01 ± 1.12	6	6.96 ± 1.23	6	8.04 ± 1.93	3.60	.450
6	3.11 ± .89	6	8.97 ± 2.49	6	13.57 ± 5.95	15.26	.299
6	3.94 ± 1.20	6	10.19 ± 3.39	5†	13.76 ± 1.55	11.87	.230
6	3.08 ± .63	7	6.44 ± 1.80	7	10.77 ± 2.28	14.39	.143
6	3.76 ± .45	7	6.44 ± 2.48	7	10.86 ± 3.33	14.67	.200
‡ 42	3.45 ± .96	44	7.66 ± 2.47	43	11.26 ± 3.81	11.22	.291

* Based on log dose-response coordinates assuming 5 μM Co^{++} equal to 1 unit response.
† One response was less than the blank, hence its value was discarded.
‡ Total.
± = stand. dev.

ether anesthesia. Twenty-four hours later, a duplicate injection was made. When cobalt was tested, it was given in 2 subcutaneous injections in 1 ml doses, in neutralized 0.15 *M* NaCl.‡ Twenty-four hours after final sample injection, 1 ml of Fe(ic)^{59} chloride (1 to 2 μc) was introduced into the tail vein. Sixteen hours after radioiron administration, a 1 ml sample of blood was taken by cardiac puncture. Activity of Fe(ic)^{59} in the blood sample was determined by counting in a well-type scintillation counter. Calculations were based on the assumption of blood volume being 5% of final body weight(3). Potencies were calculated as described by Bliss(4). The saline response did not enter into the calculations; however, only responses significantly greater than those of saline were considered valid. *Materials.* In addition to cobalt we tested plasma, phenylhydrazine-anemic plasma (5) and fractions therefrom. Most fractions were made by procedure outlined in Fig. 1(6), Two fractions in particular will be discussed: Step 1 material, which is the fraction eluted from the first DEAE-cellulose column; and Step 3 material, which is the fraction eluted from second DEAE-cellulose column. In dialyzed lyophilized form, these fractions represent yields of approximately 0.35 g and 0.06 g/l of plasma, respectively.

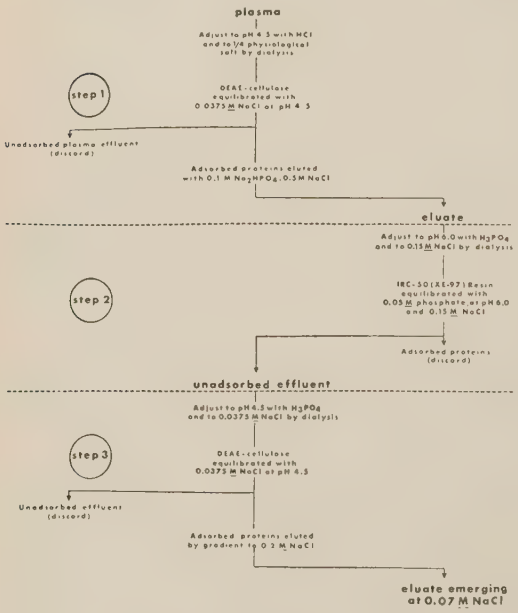

FIG. 1. Three step process for production of erythropoietin.

‡ Phosphate is omitted from NaCl used for preparation of $CoCl_2$ solution.

Results. (A) *Cobalt*: Earlier work by Goldwasser *et al.*(7,8) had indicated that cobalt stimulated incorporation of iron at dose levels of a few μmoles/rat. Preliminary experiments indicated that 5 to 10 μmole doses gave responses substantially greater than the saline blank. Table I shows typical responses to these dose levels on consecutive assays. The pooled λ§ value for assays in Table I is 0.291. Later work with our assay indicated that purified fractions from anemic sheep plasma gave slopes comparable to that of cobalt in the same response range (Table II) with λ values averaging 0.229. Cobalt therefore was accepted as the primary reference

§ λ is defined as standard deviation of a single response in terms of log-dose. It is calculated by dividing the standard deviation by the slope of the dose-response curve.

TABLE II. Analysis of Effect of Standard Sheep Plasma Erythropoietin on Fe^{59} Uptake in Fasted Rats in 7 Assays. Consecutive experiments in which both levels of sheep plasma erythropoietin standard were assayed.

Saline		1 mg standard		2 mg standard		Slope	
No. rats	% uptake Fe^{59}	No. rats	% uptake Fe^{59}	No. rats	% uptake Fe^{59}	b	Lambda
6	2.61 ± 1.67	5	6.18 ± 2.43	5	10.78 ± .73	15.24	.081
6	3.10 ± .47	6	8.52 ± .68	6	9.85 ± .24	4.41	.272
6	3.57 ± 1.42	12	9.18 ± 1.61	6	12.90 ± 4.15	12.40	.230
	*	5	8.50 ± .64	4	12.49 ± 2.85	13.28	.136
6	3.56 ± .59	5	8.32 ± 2.84	5	11.12 ± 2.69	10.62	.248
6	3.78 ± 1.00	6	7.47 ± 1.62	5	11.03 ± 2.71	11.98	.179
6	3.77 ± 1.07	6	9.82 ± 2.57	6	12.08 ± 2.48	7.53	.336
† 36	3.40 ± .86	45	8.47 ± 1.80	37	11.45 ± 2.60	9.99	.229

* One rat was eaten by the others in the group thus the responses were all aberrant.
† Total. ± = stand. dev.

standard for low level responses with the 5 μmole response designated as one unit(6). It was acknowledged that the relationship between responses of cobalt and erythropoietin fractions might not hold true at higher levels or with different injection schedules. However, it was believed that these complications could be avoided by selecting a reasonably pure erythropoietin fraction for use as a day-to-day working standard. Such a fraction would have to be carefully standardized against cobalt and then checked occasionally for maintenance of potency.

(B) *Plasma*: On the basis of the cobalt standard, plasmas from individual sheep made anemic by phenylhydrazine(5) gave potencies in the range of 0.06 to 7.4 units/ml. However, pools of plasma from 25 to 50 sheep varied only from 0.2 to 1.6 units/ml. Plasmas from normal sheep, even at maximum dose of 4 ml/rat, have not given significant responses. However, by assaying Step 1 fraction from normal plasma at high concentrations, it has been calculated that normal blood contains about 0.01 unit/ml.

(C) *Purified fractions*: Step 1 fractions have shown potencies from 0.3 to 0.9 unit/mg, and Step 3 fractions have been in the range of 1 to 4 units/mg. Both types of fraction have given dose-response slopes similar to those for cobalt in the 1 to 2 unit range. Further fractionation has given materials with indicated potencies to 50 units/mg. However, since Step 3 material was already reasonably homogeneous by ultracentrifugal and electrophoretic criteria, and since it showed no toxic effects even at very high doses, it appeared to be a suitable material for use as a working standard. Table II shows results of a series of assays on a Step 3 fraction pool. By comparing results with those of cobalt in Table I, this fraction was very nearly 1 unit/mg. It has been used as the working standard for our assays and for development of dose-response curve, Fig. 2. Stored in lyophilized form in the refrigerator, it has not shown any appreciable loss of activity over several months.

Discussion. The advantage of increased

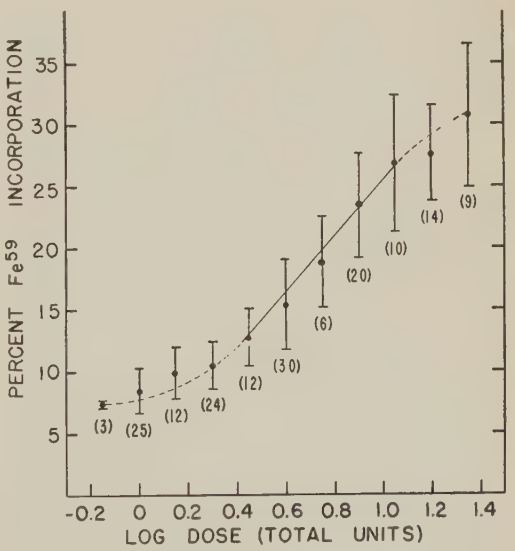

FIG. 2. Dose-response curve for standard (Step 3) erythropoietin. Data was accumulated from 7 consecutive assays, exclusive of assays used for data in Tables I and II. Figures in parentheses indicate total No. of animals assayed at a given dose level and vertical lines show stand. error of the mean. The line drawn is by inspection. One response on the 0.5 unit dose was omitted because of apparent aberrance.

TABLE III. Analysis of Dose-Response Curve.

Unit dose interval	Slope	Lambda	~ % stand. error*
1-2	6.8	.278	77.1†
2-4	16.6	.182	45.5
4-8	27.5	.139	33.1
11.2-22.4	13.3	.431	142.6

* Using 5 rats/level. Greater accuracy is naturally gained by use of more animals, thus, if 30 rats were used in the 1 to 2 unit range, the approximate stand. error would be lowered to 25%.

† Individual assays in the 1 to 2 unit range have varied in stand. error from 18 to 96%.

sensitivity of the radioiron assay in the fasted rat over that of the normal(2) or even hypophysectomized(9) rat has been demonstrated. As expected, however, degree of sensitivity in the fasted rat is a function of dose level. It is evident from Fig. 2 that the log dose-response curve shows an increasing slope, at least as high as the 11.3 unit dose (26.8% radioiron incorporation). Two other groups(9,11) working with purified urinary erythropoietin have studied the dose-response relationship in fasted rats but have not noted the sigmoid nature of the curve. An analysis of our curve between increasing dose levels (Table III) shows that the standard error of the assay can be decreased considerably at dose levels higher than the 1 to 2 unit interval. It would be more desirable to assay at these higher levels although there are several disadvantages, the most obvious being the limited amounts of erythropoietin available. Likewise, low potency materials would have to be injected in enormous volumes and/or concentrations. Under the procedure described, 4 ml of whole plasma is the practicable injection limit. Even at this concentration some mortality is incurred with resultant cannibalism, hence responses of remaining rats in the group are invalidated. The possibility of using subcutaneous dosage for eliminating some of these problems is now being considered. Korst and Bethell(10) indicate that route of administration is not critical. For purposes of following fractionation procedures, the accuracy in the 1 to 2 unit response range with 5 animals/level has proven adequate. Routinely standards are obtained at both these levels on each assay, but the samples are performed at only a single intermediate level. Critical samples, however, are generally performed at 2 levels, and reassayed if response values fall outside of limits established by the standard. It should be pointed out that curve in Fig. 2 cannot be applied directly as a standard curve for evaluation of potencies. Variations within each assay must be compensated for by performing at least 2 levels of standards in the desired response range.

Summary. Radioiron assay of erythropoietin has been analyzed and for purposes of inter-lab comparison of erythropoietic activity, the response to 5 μmoles of cobalt, given by subcutaneous injections, is suggested to represent 1 unit of activity. A house standard of partially purified sheep erythropoietin, having a potency of 1 unit/mg, has been used to establish a dose-response curve up to 22.4 units/rat. In this range the curve shows an increasing slope to 11.3 units/rat. On the basis of this assay, the potency of several fractions of sheep erythropoietin has been given.

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Clinical Studies on Erythropoietic Factor in Plasma. (25407)

KIKU NAKAO, FUMIMARO AND KUNITAKE HIRASHIMA
(Introduced by John H. Lawrence)
Gunma University School of Medicine; Professor Okinaka's Clinic, Tokyo University School of Medicine

As the result of thorough investigations on humoral regulation of erythropoiesis in recent years, it is now widely accepted that erythropoietic factor or factors are present in plasma. In investigation of factors which influence heme synthesis, it was observed that saline suspended rabbit erythroblasts incubated with plasma from rabbits submitted to intermittent anoxia or to cobalt injections showed an accelerated heme synthesis when compared with those incubated with normal rabbit plasma (1). From these results, it was postulated that such plasma factor(s) might be increased in plasma of some patients with blood dyscrasias. In this paper this plasma factor(s) in various blood diseases was studied by measuring heme synthesis in chicken erythrocytes incubated with glycine and patient plasma.

Method. Glassware was cleaned and solutions prepared with redistilled water. Glycine and delta amino levulinic acid were supplied by Daiichi Chemical Co., and radioactive iron by Oak Ridge National Laboratory. *Incubation*: Two or 6 ml of saline suspension of chicken erythrocytes was incubated with glycine ($2 - 5 \times 10^{-2}$ M), radioactive $Fe^{59}Cl_3$ (0.5 - 1.0 microcurie), and 2 ml of patient heparinized plasma, with constant agitation for 3 hours at 37°C. The mixture was then transferred to 50 ml test tube and thoroughly washed with physiological saline. The hemin was crystallized according to the method described by Chu(2). Total activity of hemin, representing total activity of radioactive iron incorporated into heme during incubation, was counted by using a well-type scintillation counter. The cyanhematin method was employed for quantitative determination of crystallized hemin. Specific activity of hemin was calculated by dividing total radioactivity (c.p.m.) of the crystallized hemin by amount of hemin in micro-Mol. The factor(s) in plasma which accelerated incorporation of radioactive iron into heme was designated tentatively as "Heme Synthesis Accelerating Factor" (H. S. A. factor). The variable activity of chicken erythrocyte suspensions in each series of experiments necessitated strict control experiments using normal plasma.

Results. When plasma from normal human beings was used, the specific activity varied $\pm$ 15%. Table I presents results of 2 experiments in which activity of H.S.A. factor (specific activity of hemin) in various diseases, is compared with normal controls. Plasma from patients with iron deficiency anemia and hemochromatosis contained less than normal H. S. A. activity, while in hemolytic anemia, polycythemia, aplastic anemia and acute leukemia the activity was increased. The increase was especially marked in the last 2 diseases.

By using chicken erythrocyte hemolysate prepared by the method of Dresel and Falk (3), and delta amino levulinic acid ($4 - 5 \times 10^{-4}$ Mol.) instead of erythrocyte suspension and glycine, H. S. A. activity was even more distinctly demonstrable in aplastic anemia and acute leukemia (Table II).

A marked difference was observed in H. S. A. activity when heparinized plasma from a patient with aplastic anemia was assayed separately by the 2 methods.

Although it seemed possible that stable iron present in plasma might interfere with incorporation of radioactive iron into heme, there was no correlation between plasma iron levels and H. S. A. activity. A nonprotein filtrate (4) prepared from plasma which was very effective in accelerating *in vitro* radioiron incorporation into chicken erythrocyte hemoglobin (last 2 patients, Table II) did not accelerate incorporation of radioactive iron into heme in incubated chicken erythrocytes.

Discussion. Specific activity of crystallized hemin indicates the effect of heme synthesizing potency of the incubation mixture plus

TABLE I. Activity of Heme Synthesis Accelerating Factor in Various Human Diseases Assayed by Employing Chicken Erythrocyte Suspension and Glycine.

Disease	Hb, %	Blood findings, R.B.C. × million	Serum Fe, γ/100 ml	S.A. of Fe^{59} in heme, cpm/μM	% of normal
(i) Using 6 ml of chicken erythrocyte suspension					
Normal	101	4.73	151	4320	100
Iron deficiency anemia	46	3.02	48	1950	37
Idem	38	3.44	60	2340	54
Hemochromatosis	83	3.88	295	2440	57
Banti syndrome	83	4.69	65	5500	130
Physiol. saline				3820	89
(ii) Using 2 ml of chicken erythrocyte suspension					
Normal	100	4.92	135	170	100
Iron deficiency anemia	58	3.80	13	80	47
Pancreas cancer	58	3.37	45	125	74
Polycythemia vera	134	7.12	83	720	430
Hemolytic anemia	65	2.86	220	840	490
Aplastic anemia	78	3.61	255	2210	1300

that of added plasma. Using hypophysectomized rats as bioassay animals, Gurney *et al.* (5) reported a high erythropoietin level in plasma and urine from patients with aplastic anemia, acute leukemia, and pernicious anemia. A high titer of erythropoietin has been found in urine of some patients suffering from aplastic anemia(6). Elevated erythropoietin activity in plasma has been observed in patients with polycythemia(7). We observed that plasma from patients with hemolytic anemia, polycythemia, aplastic anemia and acute leukemia, accelerated *in vitro* heme synthesis in chicken erythrocytes. There is no evidence to indicate that the H.S.A. factor is the same as the erythropoietin studies by other investigators, because the methods employed to assay for erythropoietin and H.S.A. factor are essentially different. Nevertheless, it is noteworthy that the H.S.A. factor was increased in diseases in which an elevated erythropoietin level is observed. Although a decreased erythropoietin level in plasma has never been detected, activity of the H. S. A. factor was somewhat reduced in iron deficiency anemia and hemochromatosis.

Since there is no cell division in peripheral erythrocytes, the effect described here must be directly on acceleration or activation of heme synthesis. Borsook *et al.*(4) described a heat stable plasma factor which is active in accelerating labeled amino acid incorporation *in vitro* into rabbit reticulocyte protein, especially hemoglobin. The activity is not destroyed or precipitated by boiling at pH 5.5. A similarly prepared filtrate from plasma with high H. S. A. activity was completely inactive. This strongly suggests that the H. S. A. factor is different in its chemical nature from Borsook's factor.

TABLE II. Activity of Heme Synthesis Accelerating Factor in Various Human Diseases Assayed by Employing Chicken Erythrocyte Hemolysate and A. L. A.

Disease	Hb, %	Blood findings, R.B.C. × million	Serum Fe, γ/100 ml	S.A. of Fe^{59} in heme, cpm/μM	% of normal
Normal	100	4.92	135	11 } Avg 9.7	100
"	95	4.87	124	8.4 }	100
Iron deficiency anemia	65	3.47	24	2.6	27
Idem	37	2.67	34	4.5	46
Secondary polycythemia*	112	6.12		51.4	530
Acute myeloid leukemia	32	1.48		148	1530
Idem	60	2.99	158	179	1840
"	45	2.19	176	262	2700
"	34	1.50	210	1260	13000
Aplastic anemia	78	3.61	255	1010	10400

* Secondary polycythemia due to cerebellar hemoangioma.

Summary. 1) A heme synthesis accelerating factor was detected in human plasma by employing a newly devised method of incubating chicken erythrocytes with glycine, radioactive iron and human plasma. Alternatively, one can use chicken erythrocyte hemolysate with delta amino levulinic acid. 2) This factor(s) was increased in hemolytic anemia, polycythemia, aplastic anemia and acute leukemia, and decreased in iron deficiency anemia and hemochromatosis. 3) "Heme Synthesis Accelerating Factor" and Borsook's factor are not identical. 4) Relationship between the "Heme Synthesis Accelerating Factor" and erythropoietin is discussed.

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Nicarbazin Induced Hypercholesterolemia in the Hen.* (25408)

HAROLD S. WEISS (Introduced by Gerald Litwack)
Department of Poultry Science, Rutgers, State University, New Brunswick, N.J.

The coccidiostat Nicarbazin (Merck) an equimolecular complex of 4,4′ dinitrocarbanilide (DNC) and 2-hydroxy-4,6-dimethylpyrimidine (HDP)(1), may, under certain conditions, depress egg production, egg size, and hatchability; and increase yolk mottling and inhibit deposition of shell pigments(2). To this list can be added induction of hypercholesterolemia, for, as outlined in this report, such an effect was observed in hens which had been fed Nicarbazin at levels high enough to inhibit egg production, but not so high as to be otherwise toxic.

Procedure. Nicarbazin was incorporated at desired concentrations into an all mash laying ration and fed to individually caged White Leghorns. The approximate composition of the diet was 15% protein, 3-4% fat from plant sources, ½% fat from animal sources and 1/40% cholesterol(3,4). Plasma cholesterol was measured at various intervals by a slightly modified Zlatkis procedure(5,3,6). In several trials pregnant mares' serum (PMS-Veterinary Gonadin Serum, Cutter Laboratories) was injected in the leg muscles of Nicarbazin-fed birds in an attempt to induce egg formation with exogenous gonadotrophins. At least 2 replicates were run for all treatments, but the results have been pooled in the Tables.

Results. Within 1-2 weeks after feeding mature hens Nicarbazin at a level between 0.04 and 0.07% of the diet, egg production decreased drastically and plasma cholesterol rose approximately 2 fold (Table I). Only a slight rise in plasma cholesterol was observed in males. By 4-8 weeks in younger females (6-8 months old) on the 0.04% drug level, egg production ceased completely and plasma cholesterol rose to levels 3 to 4 times that existing initially or in simultaneously sampled controls. This high level of plasma cholesterol was generally maintained to the 32nd week, when the longest trial was terminated. Total plasma lipids, while not measured, probably

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TABLE I. Effect of Feeding Nicarbazin on the Plasma Cholesterol of the Chicken.

Sex→	Females										Males				
Age at start→	6-8 mo						15-18 mo				6-12 mo				
% Nicarbazin→	.0 (control)			.04		.06		.04-.07			.0 (control)			.04-.06	
Wk after start	Body wt, kg	Eggs/ bird/ day*	Plas. chol., mg %	Body wt, kg	Eggs/ bird/ day*	Plas. chol., mg %	Body wt, kg	Eggs/ bird/ day*	Plas. chol., mg %	Body wt, kg	Eggs/ bird/ day*	Plas. chol., mg %	Body wt, kg	Plasma chol., mg %	Body wt, kg	Plasma chol., mg %
0	1.95	.75	211	1.88	.64	195	1.86	.72	199	1.62	.69	213	2.46	159	2.34	148
1				1.82	.67	263	1.77	.32	467	1.58	.27	401	2.42	164	2.17	203
2	2.04	.70	205	1.83	.22	507	1.72	.11	425	1.51	.03	238	2.44	177	2.12	206
4	2.04	.71	203	1.82	.00	652	1.68	.03	478	1.43	.00	189	2.43	180	2.05	207
8	2.05	.70	169	1.90	.00	961										
16	2.04	.63	227	1.87	.00	888										
32	1.90	.59	221	1.94	.00	738										
Avg No. of birds/measurement	23.8	23.8	23.8	15.3	17.0	9.2	18.8	15.0	14.2	7.2	7.2	7.0	5.4	5.4	6.2	6.2
Pooled S.E.	.060	.050	11.7	.062	.049	95.1	.035	.058	55.4	.076	.042	66.4	.076	11.1	.036	11.1

* Computed for the 7 days preceding body wt and plasma cholesterol measurements.

were also elevated as indicated by the extremely milky appearance of the plasma.

Rate of egg laying decreased even more rapidly in both younger hens on the 0.06% level, and older hens (15-18 months) on Nicarbazin levels between 0.04 and 0.07%, but plasma cholesterol did not reach as high a concentration as in the younger hens on the 0.04% dose. In fact plasma cholesterol of older hens declined by the second week and was near control levels by the 4th week. In both younger hens at the 0.06% level and in older hens, body weight fell steadily over the test period, in contrast to comparatively small changes in body weight of younger hens on 0.04% Nicarbazin.

Despite their failure to lay eggs, those birds that maintained elevated plasma cholesterol levels had normal plumage and large bright red combs, generally indicative in this breed of active ovarian function. As shown in Table II, their comb weight was equal to that of the controls. Similar observations have been made by Sherwood *et al.*(7) and Baker *et al.* (8) with respect to the appearance of hens on Nicarbazin. On the other hand there were often in these same birds external indications of non-laying such as increased yellow pigment in the beak, ear lobes, and shanks, and decreased spread between the pelvic bones. It was further observed that those Nicarbazin-treated birds in which plasma cholesterol never rose or in which it fell after an initial rise showed external signs of reproductive inactivity such as molting of plumage, decrease in size of combs (Table II) and loss of red color of comb.

In a few instances, a Nicarbazin-fed bird began to lay eggs at a slow rate after having ceased production and having maintained a high plasma cholesterol level for several weeks. In such cases plasma cholesterol always fell with the onset of egg-laying. Some birds in the 32-week-test passed through several such cycles of high and low plasma cholesterol with concomitant signs of reproductive activity and inactivity. After feeding Nicarbazin for 4-6 weeks, replacement of the drug-containing-ration by control feed resulted in resumed egg-laying(7,8) and return of plasma cholesterol to control levels in ap-

TABLE II. Effect of Injecting PMS into Nicarbazin-Fed Hens.

Treatment	Body wt, kg	Eggs/bird /day*	Plas. chol., mg %	Comb wt, g	Oviduct wt, g	Largest ova, in decreasing order, g No. 1	No. 2	No. 3	No. 4
Controls	1.88	.76	172	21.4	51.6	12.3	9.6	7.1	5.0
Nicarbazin† (active ovaries)	1.77	.00	853	23.5	29.1	1.7	1.1	1.0	
Controls + PMS‡	1.90	.09	241	26.2	55.9	15.3	14.9	13.7	12.9
Nicarbazin† + PMS‡	1.69	.00	663	20.2	31.4	1.0	.8	.6	
Nicarbazin† (inactive ovaries)	1.41	.00	228	3.1	16.0	<.1	<.1	<.1	<.1
Avg No. birds/measurement§	3.3	3.0	3.3	2.0	3.5		3.9 ‖		
Pooled stand. error§	.116	.076	63.9	1.70	4.69		1.98‖		
F ratio§	2.04	38.92¶	10.01¶	31.19¶	10.44¶		10.08¶		

* For 7 days preceding sacrifice. † .04% for minimum of 6 wk. ‡ Eight IM inj. of 50-100 units (1-2 cc) each of Gonadin Serum (Cutter Laboratories) over a 10-14 day interval. § From analysis of variance. ‖ Inactive ovary group excluded in computation. ¶ Significant at 1% level.

proximately 2-3 weeks. This was not tried on birds which had been on treatment for longer than 6 weeks.

On post mortem examination, the non-laying, high-cholesterol, Nicarbazin-fed bird presented a picture of partial reproductive activity similar to that described by Baker *et al.* (8). Yolk formation was obviously continuing, since many follicles were present which were at least 10 times heavier than those in an inactive ovary, but the largest follicles were still only about 1/10 the weight of normal ova approaching maturity (Table II). There were always present a number of small, flabby ova suggestive of loss of yolk material, although there was no free yolk in the body cavity. The oviducts of these birds looked comparatively normal, but were about half normal weight, although still twice as heavy as in birds with completely inactive ovaries (Table II).

Eight intramuscular injections of 50-100 units each of PMS over a 2-week interval did not cause eggs to be laid by the Nicarbazin-fed birds (Table II). This in itself was of little significance, inasmuch as the same PMS treatment actually inhibited egg-laying in control hens. However, on post mortem, it was apparent that PMS had induced yolk deposition in the control hens, since the usual sequential decrease in size of maturing ova was for the most part obscured. The ova of the Nicarbazin + PMS group, on the other hand, were no different in appearance or weight than those of birds treated with Nicarbazin alone (Table II).

Discussion. The accumulated evidence suggests strongly that the hypercholesterolemia induced in the hen by Nicarbazin is the result of resorption of yolk material before the ova reach ovulatory size and while they are still attached to the ovary. Further, in order to sustain the hypercholesterolemia, the reproductive system must remain functional to the extent that new ova are continulaly developing at least through the initial stages of yolk deposition. These conclusions are substantiated by the observations (a) that Nicarbazin does not induce equivalent cholesterolemia in males or in females with inactive reproductive systems; (b) that the hypercholesterolemia develops in inverse relationship to egg-laying, and once induced cannot be sustained if egg-laying recommences or the reproductive system becomes inactive; (c) that active yolk deposition through the initial stages of ova growth is seen in birds with Nicarbazin-induced hypercholesterolemia; (d) that resorption of yolk is indicated by absence of any ova approaching mature size and by presence of flabby ova.

Nicarbazin-induced hypercholesterolemia thus bears some resemblance to the heightened lipemic response Stamler *et al.*(9) obtained to dietary cholesterol in hens with ligated oviducts. These authors presumed that not only was excretion of lipoprotein through the egg prevented, but that mature yolks were being

ovulated into and absorbed from the body cavity. However, the hypercholesterolemia observed with Nicarbazin is several times greater than in the ligated-oviduct birds(9) despite a far smaller dietary cholesterol intake. In view of their low dietary cholesterol intake, the high blood cholesterol of the Nicarbazin birds must be primarily of endogenous origin, and therefore may raise some question as to the functioning of homeostatic mechanisms in the regulation of cholesterol biosynthesis in hens. If resorption of ova occurs sporadically in normal birds or can be stimulated by dietary fat manipulations, it may account for the great variability in the laying hens' palsma cholesterol, particularly the very high values sometimes encountered, (10,11), and may also explain the negative correlation reported occasionally between plasma cholesterol and egg production(3).

The failure of ova to mature under Nicarbazin treatment does not appear to be due to a relative lack of pituitary gonadotrophins (8). Exogenous gonadotrophins (PMS), while inducing growth in control ova, failed to increase the size of ova of Nicarbazin-treated birds (Table II). Further, no consistent effect on male gonads or fertility has been observed with Nicarbazin to suggest any specific effect on the pituitary(7,12,2), and in fact other than effects due to inanition no general tissue changes have been noted until feed levels reach 0.16%(12). The action of Nicarbazin at lower doses would seem to be specifically on the ovary and ova, and there are several reports that indicate that the DNC component of the drug is deposited in the yolk, and that the permeability of the vitelline membrane is altered(13,14,15).

Production of sustained hypercholesterolemia without changes in dietary lipid or protein may provide another means of studying cholesterol metabolism and the relationship of plasma cholesterol to atherogenesis. The optimum level of Nicarbazin to induce hypercholesterolemia in hens should be the lowest level which causes involution of ova without affecting other functions. Since egg production, while severely depressed, may still occur at drug concentrations between 0.02 to 0.04% of the feed(16), this range may represent a minimum. However, the age of the bird seems to be important in these responses, for the 0.04% level, which was effective in young hens appeared to depress body weight in older hens. For young hens the 0.06% level appears to be excessive as indicated by the loss of body weight.

Summary. The equimolecular complex of 4,4′ dinitrocarbanilide and 2-hydroxy-4,6-dimethylpyrimidine (Nicarbazin, Merck), when fed at 0.04% of a low-fat, low-cholesterol diet to 6-8 month-old laying hens, doubled plasma cholesterol within 2 weeks and tripled it within 4 weeks. Thereafter the hypercholesterolemia was maintained at levels between 600 and 900 mg % for as long as 32 weeks. The hypercholesterolemia appears to be induced and sustained by continuous resorption of yolk from partially developed ova, while yolk material continues to be deposited in other ova just beginning the growth cycle.

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Thromboplastin. I. Phospholipid Moiety of Thromboplastin.* (25409)

KARL H. SLOTTA (Introduced by George T. Lewis)
Depts. of Biochemistry and Medicine, University of Miami Medical School, Miami, Fla.

Howell(1) and McLean(2) established that the phospholipid factor necessary for formation of thromboplastin is an unsaturated cephalin. The question, however, remained whether PE† by itself, or PS by itself, their mixtures, or mixtures with lecithin are necessary. Poole and Robinson(3), O'Brien (4), and Rouser, White and Schloredt(5) maintain that PE is the active substance. Fischer and Hecht(6), however, showed that the purest PE is actually inactive. Even more confusing are opinions on activity of PS. According to Maltaner and Rapport(7), PS is active if rabbit, not avian plasma, is used in the test. Barkhan, Newlands and Wild(8) concluded from experiments on human brain PLs that PS is probably an inhibitor. Barkhan, Silva da Costa and Tocantins(9) found that PS is clearly inhibitory in buffered, saline suspension. Silva, Turner and Tocantins(10) assayed PS as an anticoagulant by using Na deoxycholate in the test suspension. Contrary to these results, Troup and Reed (11) found that of various PLs only PS gives maximal thromboplastic effect. Marcus and Spaet(12) confirmed this, stating that PS isolated from platelets could replace the platelets in thromboplastin generation. Several authors claimed that the coagulation activity is not exercised by a single PL, but by interaction of at least 2. The following authors state that either presence of lecithin is indispensable, or that lecithin increases activity of the corresponding cephalin fractions: Zunz and La Barre(13) "cytozymin"; Gracia and Levene(14) cephalin; Rapport(15) a cephalin fraction consisting mostly of PS; Therriault, Nichols and Jensen(16) very pure PS.

Methods and materials. Testing. One reason for the diametrically opposite results of different authors is the fact that they used entirely different and often dubious test methods. We found that the original Thromboplastin Generation Test of Biggs, Douglas and McFarlane(17) gives a reliable picture of activity of PL preparations, and this method was, therefore, used exclusively. PLs used, raw lecithin, protagon, and cephalin were prepared from fresh calf brain according to Klenk and Böhm(18) and Debuch(19). Lecithin was purified by chromatography on alumina(20), and cephalin was separated into 5 fractions by the method of Folch(21). Fraction III (molar ratio N : P = 0.95) contained not only PS, but also small amounts of PE, lyso-PE, mono phosphatidyl inositol, and diphosphatidyl inositol, as described by Therriault, Nichols and Jensen(16). Fraction IV (molar ratio N : P = 1.1) contained PE and PS in the proportion of 4 : 1, whereas fraction V (molar ratio N : P = 1.1) was almost pure PE with a trace of PS. Total PLs from egg were prepared from fresh egg yolks by the method of Rhodes and Lea(20). The PLs were dialyzed, and the dialyzate contained comparatively large quantities of free ethanolamine and O-phosphoryl serine, but no serine. Upon evaporation, the dialyzate left a residue which was chromatographed on silicic acid paper with phenol-water. Three ninhydrin-positive spots with Rf = 0.05 (= O-phosphoryl serine), 0.16 (= glyceryl phosphoryl serine?) and 0.73 (= ethanolamine) were obtained. We found in the total egg, PLs

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† Abbreviations used are: PL, phospholipid; PE, phosphatidyl ethanolamine; PS, phosphatidyl serine.

after dialysis 18.2% PE + lyso-PE. This corresponds to the 17.5% isolated by Rhodes and Lea(20). *Analysis of ethanolamine and serine.* The figures given above for PE and PS content of PLs we used are not absolutely correct, because the composition of the fatty acid side chains is variable. With the most recent methods of Nojima and Utsugi(22), and Dittmer, Feminella and Hanahan(23), as well as with a new method described elsewhere, we obtained good results with mixtures of pure ethanolamine and serine, but only dubious values with natural animal and especially with vegetable PLs. Thus, our values for ethanolamine and serine are only approximate. *L-α-(Dioleoyl)-PE,* obtained from Dr. Erich Baer, Toronto. By ascending chromatography on silicic acid paper with di isobutylketone, acetic acid, and water (40 : 25 : 5) only one spot, Rf = 0.73, was detectable with ninhydrin, $FeCl_3$ + sulfosalicylic acid, Sudan Black B, or Rhodamin G 6. The iodine numbers obtained by using 0.5, 1, 1, 1.5, 1.5 mg were respectively 51.3, 52.6, 54.6, 53.3, 50.8 (theor. 68).

Results. When suspensions of platelets or total brain lipids were tested in the Thromboplastin Generation Test, those containing 100 μg PLs/ml were most active. This means that the incubation mixtures contained 25 mg% PLs as the optimal concentration. In these experiments, after 3 minutes incubation an average coagulation time of 10 seconds was observed.

In all experiments serial dilutions of PL suspensions from 1,000 to 10 mg/ml were tested. Our report presents only results with levels of 100 mg/ml.

Synthetic PE is very difficult to emulsify in saline or in 0.3% pluronic F 68 acid (a polymer of ethylene sulfate). However, excellent and very stable emulsions were obtained with lecithin (1 : 1) in saline or imidazol buffer, pH 7.2. But even these emulsions had no activity (Table I, 1-4). Our total PL preparation from fresh eggs could be easily emulsified and was also inactive (Table I, 5). Fraction V certainly lacked the activity of whole platelet or brain PLs, but it was more active than the synthetic PE or egg PLs (Table I, 6). That fraction V was not completely inactive appears due to a minute amount of PS which it contains. It was demonstrated as follows that this may actually be the case: Chloroform solutions of different quantities of fraction III (mainly PS) were evaporated together with those of synthetic PE, and these mixtures of 2, 5, 10, and 15% PS with PE were tested in saline (Table I, 7-10). Even though these percentages of PS may not be quite accurate, due to difficulties of analytical determination of serine in PLs, we may conclude that fraction V contained about 2% PS (corresponding to 0.13% serine). Furthermore, it is evident that mixtures containing about 10% PS with PE are the most active. Fraction IV with 20% PS (Table I, 11) is more active than fraction V (Table I, 6). However, higher concentrations of PS than contained in fraction IV do not enhance activity, since fraction III (Table I, 12) is even less active than fraction IV (Table I, 11). A mixture of 3 parts of fraction III with 7 parts of fraction V gives practically the same coagulation time as fraction IV, because both contain about 20% PS in 80% PE (Table I, 13). Another preparation gave practically the same result (Table I, 14), and was also highly ac-

TABLE I. Activity of PS in Mixtures with PE. (Coagulation time in sec.)

Exp. No.	Incubation time in min. 1	2	3	4	5	6
1	160	142	138	135	130	115
2	160	138	135	125	100	95
3	220	210	130	125	130	102
4	102	100	95	89	65	48
5	68	59	43	39	40	54
6	27.5	17	16	20	20.5	25
7	22	15	14.5	17.5	18.5	19
8	12	11.5	12	13	15	16
9	11	8.5	10.2	12	13	15
10	12	11.5	10.5	11	12.4	14
11	14	9.5	10	10.2	11	15
12	14	11.2	12.5	13.8	13.9	14.5
13	9.2	8.2	7.5	9	11.5	12.8
14	9	8	7.5	6.4	8.5	12.5
15	15	9.5	10.8	14	17.5	18

1. Synthetic L-α-(Dioleoyl)PE in saline. 2. Same plus lecithin (1:1). 3. Same plus lecithin (1:1) in imidazol buffer. 4. Same in 0.3% pluronic F 68 acid. 5. Egg PLs. 6. Folch V. 7. 2% PS (brain), 98% synthetic PE. 8. 5% PS (brain), 95% synthetic PE. 9. 10% PS (brain), 90% synthetic PE. 10. 15% PS (brain), 85% synthetic PE. 11. Folch IV (20% PS). 12. Folch III (70% PS). 13. Folch III + V (3:7). 14. Repetition of 13 with new preparation. 15. Same as 14, diluted 1:10.

tive at levels as low as 10 μg/ml (Table I, 15).

Separation of PE from PS is difficult, particularly when only traces of PS are present. This appears to be one of the reasons why PE has often been taken for the thromboplastin PL factor. In the yolk of fresh eggs there is no PS. In agreement with this we found no thromboplastic activity, contrary to findings of others(3).

Lecithin is inactive (Table II, 1) as are also mixtures of lecithin with synthetic PE (Table I, 2, 3). Mixtures of lecithin and our fraction V (Table II, 2) have the same minimal activity as fraction V component alone. Lecithin, however, is known to influence strongly the activity of PS, as Therriault, Nichols and Jensen(16) have shown. It must be emphasized that optimal concentrations for mixtures of PS with lecithin (phosphatidyl choline), as well as PS with PE, are the same, namely, around 10% PS (Tables II, 4 and I, 9).

This fact supports the theory that the active PL is not phosphatidyl choline, PE, or PS alone, but a complex existing in nature or formed in organic solvents in stoichiometric proportions. PLs from platelets and brain contain PS, PE, and phosphatidyl choline in optimal proportions(12,24) and, therefore, possess highest activity.

As Table III shows, very active preparations, *e.g.*, of mixtures of 20% PS in PE, are not completely inactivated, if less than 1 mg of sodium deoxycholate is present/mg PL. Test suspensions become clear, because the well-known formation of deoxycholate complexes with fatty acid groups takes place.

TABLE II. Activity of PS in Mixtures with Phosphatidyl Choline. (Coagulation time in sec.)

Exp. No.	Incubation time in min. 1	2	3	4	5	6
1	75	50	39.6	36	34.5	37
2	27	17.8	17.5	16	21	24.5
3	10.5	8.8	9.8	11	12	12.5
4	9.5	8	8.8	9.8	11.8	14
5	12	11.5	10.5	11.5	12.4	14
6	11.8	11.5	13	13.8	15	20

1. Lecithin. 2. Lecithin + Folch V (1:1). 3. Lecithin + 5% PS (brain). 4. Lecithin + 10% PS (brain). 5. Lecithin + 15% PS (brain). 6. Lecithin + 20% PS (brain).

TABLE III. Inhibition of PS + PE Mixtures by Sodium Deoxycholate. (Coagulation time in sec.)

Exp. No.	Incubation time in min. 1	2	3	4	5	6
1	26	13	8	8	9	9
2	80	60	24	14	9	9
3	47.5	35	19	11	10.5	13
4	40	45	28	15.5	10	9.4
5	360	110	95	105	100	90
6	90	85	90	90	91	88
7	120	102	95	95	93	90
8	100	88	105	100	85	93
9	130	125	125	120	130	133.5

Suspensions in imidazol buffer pH 7.2 of 1 mg/ml of fraction III + V (3:7) were used containing the following amounts in mg of sodium deoxycholate per ml of suspension: (1) none, (2) 0.2, (3) 0.5, (4) 1.0, (5) 2.0, (6) 3.0, (7) 4.0, (8) 5.0, (9) 6.0.

Generation of thromboplastin only is retarded, but after 5 to 6 minutes a potent thromboplastin is formed (Table III, 1-4).

If, however, 2 to 6 mg sodium deoxycholate/mg PL is used, pronounced and practically the same inhibition effects occur (Table II, 5-9). The threshold value lies between 1 to 2 mg deoxycholate to 1 mg PLs. Apparently below this ratio less than one deoxycholate is available for each fatty acid ester in the PLs. Evidently, a 1 : 1 relationship is necessary for maximal inhibitory effect.

Summary. Phosphatidyl ethanolamine and phosphatidyl choline are inactive in the Thromboplastin Generation Test, but they become highly active when present with about 10% phosphatidyl serine. Phospholipids from fresh eggs are inactive, because they contain no phosphatidyl serine. Sodium deoxycholate gives clear, but less active or inhibitory suspensions.

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Effect of Methyl Linoleate on Tissue Cholesterol of Normal and Vit. E-Deficient Rabbits.* (25410)

L. C. SMITH, Y. S. SHIN AND D. T. FREIER

Dept. of Biochemistry, School of Medicine, State University of South Dakota, Vermillion

It has been shown(1,2) that a diet high in polyunsaturated fatty acids could significantly lower serum cholesterol; whereas Vit. E-deficient diet increased serum and muscle cholesterol(3,4). Shull *et al.*(5) have shown that supplementation of Vit. E-deficient rations with certain antioxidants delayed onset of dystrophy symptoms, but did not prevent the rise in plasma and muscle cholesterol. It appears, therefore, that Vit. E and unsaturated fatty acids might be interrelated with one another in controlling cholesterol levels of various body tissues. Previous studies on the effect of Vit. E or essential fatty acids on serum or tissue cholesterol level have been made with either Vit. E or essential fatty acids alone. These have been further complicated by simultaneous feeding of cholesterol or other antioxidants. Our studies describe the effect of feeding Vit. E and methyl linoleate on cholesterol content of serum, liver, kidney, heart, spleen, brain and muscle of normal and Vit. E-deficient rabbits.

Methods. Forty-two rabbits of both sexes were divided into 4 groups and fed the following diets *ad lib.*: A. Dystrophy producing diet of Young and Dinning(6); B. Dystrophy producing diet plus 10% methyl linoleate; C. Dystrophy producing diet plus 8 mg oral alpha tocopherol acetate thrice weekly; D. Dystrophy producing diet plus 8 mg oral alpha tocopherol acetate thrice weekly plus 10% methyl linoleate. When methyl linoleate was incorporated into the diet, an equivalent weight of sucrose was deducted. After symptoms appeared(7), blood was withdrawn by heart puncture and animals were sacrificed by intravenous injection of air. Then aliquots of spleen, kidney, liver, heart, brain and muscle were removed, and approximately 100 mg (wet wt) were used for cholesterol analyses. Cholesterol was determined by the method of Herrmann(8) after slight modifications suggested by the author.

Results. Distribution of cholesterol in vari-

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TABLE I. Effect of Diet on Cholesterol Content of Rabbit Tissues. Values expressed as mg % cholesterol and probable error of mean.

Tissue	Diet A Vit. E defic.	Diet B Vit. E defic. + meth. lin.	Diet C Vit. E defic. + vit. E	Diet D Vit. E defic. + meth. lin. + vit. E
Serum	362 ± 19.7	230 ± 8.7	178 ± 9.4	147 ± 15.5
Spleen	426 ± 7.5	355 ± 12.2	388 ± 12.2	357 ± 7.8
Kidney	386 ± 11.2	328 ± 7.0	335 ± 11.4	346 ± 12.5
Liver	382 ± 15.3	294 ± 6.5	341 ± 24.9	343 ± 3.4
Heart	175 ± 5.4	142 ± 4.5	136 ± 4.9	147 ± 6.4
Brain	1531 ± 63.4	1509 ± 50.4	1406 ± 45.8	1580 ± 37.1
Muscle	173 ± 3.1	166 ± 5.1	71 ± 3.4	76 ± 9.9

ous tissues of normal and dystrophic rabbits is shown in Table I. Values are expressed as mg cholesterol/100 g wet tissue or 100 ml serum, and represent average of tissues from 10 animals each on Diets A & B, and 11 rabbits each on Diets C & D. There was a significant decrease in serum cholesterol ($p < 0.01$) of animals receiving dystrophic Diet B plus 10% methyl linoleate as compared to those on dystrophic Diet A alone. Less significant differences were noted in liver, heart, kidney and spleen ($p < 0.01$), while brain and muscle failed to show important variations in the 2 groups. When animals of Group A were compared to Group C, marked lowering in cholesterol of the latter group was noted in serum and muscle ($p < 0.01$) with less but significant difference in heart tissue ($p = 0.01$). The other tissues showed lower, but not significant differences. Tissue cholesterol of rabbits on Diet C did not vary from those receiving methyl linoleate on Diet D. Rabbits on Diet C had similar tissue cholesterol values to those receiving dystrophic Diet B supplemented with methyl linoleate. The exceptions were that serum cholesterol of the latter group had a slight increase ($p > 0.01 < 0.05$) while muscle cholesterol showed a marked elevation ($p < 0.01$).

Discussion. It has been previously shown (3,4) that diets deficient in Vit. E cause increased skeletal muscle and serum cholesterol, and our work substantiates these findings. Studies in several laboratories have established the effects of unsaturated fats in lowering serum cholesterol(1,2). Our data do not show that animals receiving Vit. E and 10% methyl linoleate have significantly lower serum or tissue cholesterol than animals receiving Vit. E alone. This may be explained by the fact that the latter group of animals did not receive an isocaloric substitution of hydrogenated oil as did those of other workers (9).

Perhaps the most important point from these experiments is that rabbits on a dystrophic diet with methyl linoleate had lower serum and tissue cholesterol than animals receiving the dystrophic ration alone. Only muscle and brain showed no decrease in cholesterol. Also rabbits fed methyl linoleate supplemented dystrophic diets had similar tissue cholesterol values to animals receiving Vit. E, muscle being the one exception. From these observations, it is concluded that Vit. E is a more important factor than methyl linoleate in maintaining normal tissue cholesterol metabolism, but methyl linoleate may aid in reducing tissue cholesterol, muscle excepted, when Vit. E stores are inadequate. It is conceivable that the beneficial effects of methyl linoleate in lowering tissue cholesterol may be due in part to the fact that it spares traces of Vit. E in the diet.

Summary. 1. Distribution of cholesterol in 7 tissues of normal and dystrophic rabbits was determined. 2. Significant decreases in serum, liver, heart, kidney, and spleen cholesterol occurred in animals receiving dystrophic diet plus 10% methyl linoleate as compared to those receiving dystrophic diet alone. 3. Vit. E caused a lowering of cholesterol in serum, muscle, and heart tissue. 4. 10% methyl linoleate did not significantly lower tissue cholesterol of normal animals. 5. Dystrophic rabbits receiving methyl linoleate had similar tissue cholesterol to normal rabbits with the exception of muscle tissue.

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Hormonal Augmentation of Antimetabolite Chemotherapy.* (25411)

DANIEL S. MARTIN, L. S. DIETRICH AND RUTH A. FUGMANN

Depts. of Surgery and Biochemistry, University of Miami, School of Medicine, Fla.

Our emphasis has been placed on utilizing combinations of drugs in treatment of neoplastic growth(1-5). Previous reports have shown mouse mammary adenocarcinoma 755 to be adversely affected by stilbestrol(5), and by the niacin antagonist, 6-aminonicotinamide (3). The present studies demonstrate the effects of the combined administration of the hormone and the anti-vitamin on the 755 tumor.

Materials and methods. Mammary adenocarcinoma 755 grown in the C57 Bl mouse was the tumor-host system used in all studies. The tumor was implanted into the right axillary region of the mouse by the usual trocar technic. The animals were 2-4 months old, weighed 18-22 g and were housed in plastic cages in an air-conditioned, constant temperature room (74°F). They had free access to Rockland pellets and water. 6-Aminonicotinamide (6-AN), in saline, was administered intraperitoneally at a dose of 2 mg/kg/day; stilbestrol and testosterone, in sesame oil, were administered intramuscularly at doses of 10 mg/kg/day and 25 mg/kg/day respectively. *Biological.* Treatment was initiated on very well-established tumors (14-20 days old) and was continued for 5 to 7 days. In order to note any delayed host toxicity, a waiting period of 5 to 6 days of no treatment was observed after last injection. At the end of this period the animals were sacrificed, the tumors were removed and wet weights were determined to the nearest milligram. A group of animals (labelled "sacrificed controls") was sacrificed on the day that treatment of the remaining groups was initiated. Mean tumor weight of this group served as a reference point for evaluating the effect of chemotherapy on other groups in the experiment. The statistical method used to calculate the significance of the results has been reported(3). *Enzymatic.* The animals were sacrificed by cervical rupture 24 hours after cessation of therapy. The tissues were immediately removed, chilled in crushed ice, blotted dry, and homogenized in 0.25 M sucrose containing 0.02 M nicotinamide and 0.004 M versene in a glass homogenizer of the Potter type(6). The ability of tissue homogenates to convert to citrate was measured as follows: pyruvate, 0.011 M; ATP, 0.007 M; $MgCl_2$, 0.008 M; nicotinamide, 0.04 M; potassium phosphate buffer (pH 7.4), 0.001 M; fluorocitrate, 2.15 x 10^{-4} M; and the appropriate volume of tissue homogenate was added to ice-cold reaction vessels. Ice-cold isotonic KCl was added to give, after addition of substrate, a final volume of 2.7 ml. Immediately before the vessels were placed in a constant-temperature water bath, 0.2 ml of a 0.10 M malate solution was added. All reactants were neutralized with KOH prior to addition. Incubation was carried out at 37°C with agitation for 30 minutes, at which time the reaction was stopped by addition of 0.5 ml of 30 trichloro-

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TABLE I. Effect of Androgen-Estrogen Therapy on 6-Aminonicotinamide Carcinostasis.

Exp.	Sex	Group*	Mean tumor wt ± S.E. (mg)	No. animals dead/total	% change in body wt
1	♂	Sac. control	463 ± 64		
		6-AN	1009 ± 159	0/20	− 3
		" + Test.	639 ± 129	1/20	+ 8
		Test. + Stilb.	2632 ± 294	1/20	+20
		6-AN + "	526 ± 133	5/20	− 6
		" + Test. + Stilb.	207 ± 45	0/20	− 5
2	♀	Sac. control	405 ± 57		
		6-AN	798 ± 157	0/15	0
		" + Test.	430 ± 105	0/17	+ 7
		Test. + Stilb.	2179 ± 114	0/18	+24
		6-AN + "	300 ± 94	1/16	− 6
		" + Test. + Stilb.	188 ± 46	0/16	0

* Sac. control = Animals sacrificed on day injections begun to other groups. Therapy was begun upon well-established tumors (14-20 days old). 6-AN = 6-aminonicotinamide (2 mg/kg daily); Test. = Testosterone (25 mg/kg daily); Stilb. = Stilbestrol (10 mg/kg daily).

acetic acid (TCA). Citrate analyses(7) were carried out on the supernatant material obtained upon centrifugation. 100 mg of tumor tissue, as homogenate, was added to each reaction flask.

Results. Biological studies. The results of 2 representative experiments are noted in Table I. The chart graphically summarizes all 8 experiments.

The combination of 6-AN and stilbestrol produced a significantly greater anti-tumor effect than either agent administered alone in 3 out of 4 experiments in males and in 3 out of 4 experiments in females. The 4th experiment in males showed borderline significance; the 4th experiment in females was negative.

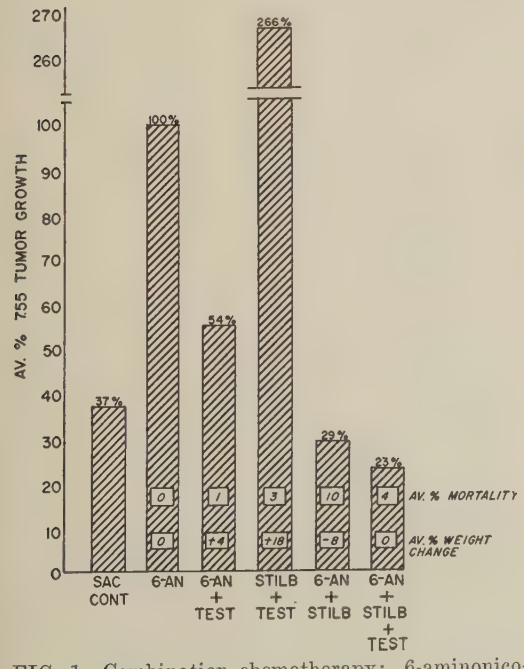

FIG. 1. Combination chemotherapy: 6-aminonicotinamide (6-AN) + stilbestrol (stilb) + testosterone (test). (Summary of 8 experiments, 4 ♂ + 4 ♀.)

TABLE II. Effect of Diethylstilbestrol and 6-aminonicotinamide Administration on Conversion of Malate to Citrate in 755 Tumor Homogenates.

Exp.	Treatment*	Activity,† μmoles citrate produced/30 min./g tissue wet wt
1	Saline control	8.4 ± .4 (9)
	Stilbestrol	8.3 ± .4 (10)
2	6-AN	8.5 ± .3 (8)
	" + stilbestrol	4.7 ± .4 (8)

* 6-aminonicotinamide was administered at level of 2 mg/kg for 6 consecutive days. Stilbestrol was given in sesame oil at 10 mg/kg/day.

† ± stand. error. Figures in parentheses represent No. of tumors analysed.

Addition of testosterone to 6-AN + stilbestrol maintained the anti-tumor effect of the combination of 2 drugs and at the same time markedly reduced host toxicity.

Addition of testosterone to 6-AN resulted in increased carcinostasis over that produced by 6-AN alone. Degree of carcinostasis, however, was not of a statistically significant magnitude in females and only of borderline significance in males.

Biochemical studies. Stilbestrol administration *per se* had no effect on ability of 755 tumor homogenates to convert malate to citrate (Table II, Exp. 1). Stilbestrol, however, markedly enhanced ability of the niacin an-

tagonist, 6-AN, to antagonize conversion of malate to citrate (Table II, Exp. 2). This level of 6-AN (2 mg/kg), by itself, has been shown to have no effect on the malate to citrate pathway(8). Previously, lethal levels of 6-AN were necessary to demonstrate a significant antagonism of the malate to citrate system in 755 tumor homogenates(8). Thus, stilbestrol, by reasons unknown, increases 6-AN toxicity to the 755 tumor without exerting the same degree of augmentation of toxicity to the host tissue. These preliminary enzymatic studies are correlated with the biological findings (Table I). How these findings are related to those of Ville *et al.*(9) and Hurlock and Talalay(10), who have previously shown a relationship between estrogens and pyridine nucleotide metabolism, is unknown.

Summary. 1) The data show that combination of hormone, stilbestrol, and anti-vit, 6-aminonicotinamide, has a greater anti-tumor effect than that produced by either drug alone. Further, addition of testosterone to the combination lessens host toxicity without altering the anti-tumor effect. 2) At an enzymatic level stilbestrol enhanced ability of this niacin antagonist to antagonize conversion of malate to citrate, suggesting a possible mechanism of action to explain the observed biological facts.

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Reversible Inhibition of 2,5-Alkyl Benzimidazoles on Chicken Sperm.* (25412)

UNABELLE BOGGS BLACKWOOD† AND GROVER C. HARRIS, JR.
(Introduced by C. S. Shaffner)
Dept. of Poultry Husbandry, University of Maryland, College Park

Alkyl benzimidazoles are known inhibitors of a variety of biological systems exhibiting a characteristic order of inhibition(1-6). Because of the nature of inhibition of virus multiplication and the characteristic inhibitory order, it has been suggested that the benzimidazoles inhibit a basic biosynthetic mechanism(s) involving nucleic acids and/or nucleoprotein(2,3,7). With these suggestions in mind, studies presented here were conducted to determine the effect of three 2,5-alkyl benzimidazoles on chicken sperm, a relatively simple biological system whose metabolic processes were assumed to involve mostly respiration.

Materials and methods. Pooled samples of semen were used. Semen was collected from Univ. of Maryland flightless strain males by massage technic. 2,5-Dimethylbenzimidazole (DMB) and 2-ethyl-5-methylbenzimidazole (EMB) were dissolved in 0.07 N HCl and 2-hepta-5-methylbenzimidazole (HMB) was

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† Present address: Poultry Science Dept., Ohio State Univ., Columbus.

dissolved in 0.118 N HCl at concentration of 10 mg/ml. Aliquots were added to 1/6 M phosphate buffer(8) to which semen was added at 1:9 dilution for motility determinations and 1:3 dilution for storage and fertility determinations. For fructose fermentation studies, as indicated by acid production, aliquots of inhibitor solutions were added to ⅓ M solution of fructose diluted with ⅙ M phosphate buffer giving final concentration of 2.25 mg/ml of fructose. Three parts of this solution were mixed with 1 part semen. These values were based on previous studies using pH depression as indicator of carbohydrate utilization(9). Acid production was determined, after incubation in stoppered tubes for 3 and 6 hours in 37°C water bath, by titration to pH 8 with 0.02 or 0.05 N NaOH. For fertility determinations, the semen-inhibitor-buffer solution was stored 3 days at 10°C. The inhibitor was essentially removed by diluting to 1:23 and 1:39 with buffer, centrifuged, and resuspended to original volume of 1 ml with Lake's solution(10). This sperm suspension was then inseminated into hens taking care not to exceed 1½ hours for washing and insemination process. The number of hens inseminated varied between 5 and 10/treatment. Controls were: a) fresh semen inseminated on day of collection to test fertilizing capacity of untreated semen, and b) stored semen diluted 1:3 with phosphate buffer minus inhibitors. Motility was determined 2 hours after semen was added to inhibitor-buffer solution and scored subjectively from 0 to 4 (0 = no motility; 4 = motility comparable to freshly collected semen). Removal of inhibitor in motility reversal studies was conducted in the same manner as in fertility studies except that sperm were held at room temperature 2 hours, diluted to 1:39 only, and resuspended to 1:9 dilution.

Results. Table I shows that the order of inhibition of chicken spermatozoa motility by benzimidazoles was DMB<EMB<HMB. The same inhibitory order was also found in fructolysis; DMB failed entirely to affect acid production and only doses of 300 μg or more of EMB were effective, while each dose of HMB (molar equivalents of 50, 100, and 200 μg EMB) progressively decreased acid production. It is evident that levels of DMB and EMB which completely inhibited motility had no effect on fructolysis. Motility was restored by simply washing the inhibited sperm with phosphate buffer. Restoration was complete for all cases except those levels which also inhibited fructose fermentation, *i.e.*, 300 and 500 μg EMB and each level of HMB (Table I). An additional washing of 300 μg EMB completely restored motility and increased that of 144 μg HMB from 2 to 3.

After 3 days storage of semen with doses of inhibitors, which partially inhibited motility but had no effect on acid production, fertility was greater than that of controls receiving no inhibitors (Table I). However, in cases in which inhibitor levels decreased acid production, fertility was decreased to 0. Thus, no fertility was obtained when HMB was added, since each dose decreased acid production. The relationship of fertility enhancement to motility inhibition was different for DMB and EMB; *i.e.*, fertility enhancement by DMB was directly related to motility inhibition, but for EMB these factors were inversely related. The increase in dilution from 1:23 to 1:39 of EMB-semen suspension with phosphate buffer for removing the inhibitor after storage appeared to increase fertility.

Discussion. The greater activity of EMB over DMB in inhibiting sperm motility and fructolysis is of the same inhibitory order as that found in other biological systems(1-6) thus adding further support to the suggestion that these compounds inhibit some basic metabolic mechanism(3). However, the marked increased inhibition of HMB over EMB suggests a difference in inhibitory action of 2-alkyl benzimidazoles (having alkyl chains longer than 2 carbons) on unicellular organisms as compared to multicellular ones since lengthening the chain past 2 carbons increased inhibition in bacteria(1) but failed to do so in chick embryos(4) or in virus multiplication on chick chorioallantoic membrane(2). This difference may be related to increased solubility in lipids with increased chain length, cell surface, and cell penetration.

The inhibitory action on motility of each level of inhibitors could be completely or partially reversed, depending upon inhibitor level,

TABLE I. Effect of Three 2,5-Alkylbenzimidazoles on Motility, Fructolysis, and Fertility of Chicken Sperm.

Inhibitor	μg/ml (total sol.)	Motility after 2 hr		Fructolysis* (ml)		Fertility after 3 days storage 1:23†		1:39†	
		Unwashed	Washed	After 6 hr, .05 N NaOH	After 3 hr, .02 N NaOH	% fert.	No. eggs	% fert.	No. eggs
Fresh semen						93	30	86 (80-90)‡	72
DMB	0	4	4	3.50	3.80			12 (5-19)	43
	100	3+	4	3.55	4.03			24 (18-30)	55
	200	3	4	3.51	3.93			26 (13-39)	51
	300	2	4	3.53	4.19			57 (40-74)	43
	400	1	4	3.43	4.42			60 (54-66)	48
	500	0	4		4.35				
EMB	0	4	4	4.35	4.00	8 (3-13)‡	45	5	20
	50	3	4	4.54	4.08	27	22	48 (43-52)	44
	100	2+	4	4.48	4.16	29 (13-44)	41	25	12
	200	0	4	4.39	4.79	0	48	5	19
	250	0	4	4.33		0	18		
	300	0	3	4.02	3.12	0	25		
	500	0	2	3.41	3.40				
HMB	0	4	4	4.42	3.90				
	72	0	3		3.41			0	29
	144	0	2	3.91	2.82			0	25
	288	0	2	3.28	2.12			0	27

* Acid production values corrected by subtracting quantity of acid added with inhibitors; 1 ml semen was used in 6 hr incubation and 0.5 ml used in 3 hr.

† Total dilution of semen after storage to remove inhibitors.

‡ Range of 2 exp. in parentheses.

by simply diluting out the inhibitor with buffer. The fact that fertilizing capacity was maintained after 3 days storage indicates that sperm life is prolonged by these inhibitors and that after removal they have no obvious, adverse after-effects. The highest average fertility results (48-60%) after 3 days storage are considered quite good for laying hens nearing completion of first year of production. Successful storage of chicken semen for 3 days has not previously been reported. Therefore, use of benzimidazoles or perhaps other inhibitors may permit successful extension of storage period beyond 3 days.

The ease with which these inhibitors are removed by dilution and the apparent freedom from after-effects has previously been reported on experiments in virus interference (7) and inactivation of newt larvae(6).

The fact that motility is completely inhibited by levels of DMB and EMB which have no effect on fructolysis indicates that these compounds are able to inhibit some metabolic process or mechanism which is intimately concerned with protein contraction and is independent of fructolysis. Prolongation of fertilizing ability is also independent of fructolysis. This conclusion is substantiated by the actual determination of fructose disappearance (Harris, unpublished) which showed that inhibitory levels of DMB and EMB which allowed maximum fertility had no effect on fructose utilization. Indeed normal fructolysis appears to be necessary during storage for maintenance of generative powers since levels which decreased acid production completely inhibited fertilizing capacity.

The exact process(es) interrupted by the

benzimidazoles is unknown but is undoubtedly complex. It has been suggested that they interrupt some biosynthetic mechanism involving nucleic acid and/or nucleoprotein(2, 3,7). The particular metabolites which are capable of partially reversing inhibition of certain benzimidazoles in various biological systems support this view: Vit. B_{12} is active in chick embryos(4), in *E. histolytica*(11), and in *E. gracilis*(12); adenine and guanine in other microorganisms(13); flavinadeninedinucleotide and riboflavin phosphate in azo dye destruction by liver homogenates(5); and glycine in delta-amino-levulinic acid synthesis(14). If these compounds interrupt some biosynthetic mechanism at levels which prolong fertilizing ability and inhibit sperm motility, it would seem to follow that motility may be dependent upon this same aspect of biosynthesis and that its inhibition may prolong viability of sperm.

Summary. Motility, fructolysis, and fertilizing capacity of chicken sperm were determined following their subjection to various dose levels of 2,5-dimethyl-(DMB), 2-ethyl-5-methyl - (EMB), and 2 - hepta - 5 - methyl-(HMB) benzimidazoles. Motility after 1-2 hours and acid production from fructose after 3 and 6 hours were inhibited by the compounds with the following order of inhibition: DMB<EMB<HMB. Motility was completely inhibited by levels of DMB and EMB which had no effect on fructolysis. Fertilizing ability was prolonged by inhibitor levels which did not reduce fructolysis indicating that the disturbed metabolic process which inhibited motility was responsible for prolongation of fertilizing capacity and that this disturbance is not intimately concerned with fructolysis. The storage period for chicken semen was extended to 3 days with excellent fertility obtained with both DMB and EMB. The ease with which these compounds are removed by dilution and the lack of adverse after-effects appears to make them peculiarly useful for preservation of chicken sperm or possibly other types of cells or tissues.

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Influence of Hypophysectomy on Porphyrin Synthesizing Enzyme Activity of Rat Harderian Glands and Livers.* (25413)

Frank H. J. Figge and Roger H. Davidheiser
Dept. of Anatomy, University of Maryland School of Medicine, Baltimore

It was previously shown that extracts of Harderian gland and liver homogenates of normal rats exhibit a high porphyrin-producing enzyme activity(1), and that activity of the Harderian gland extract was considerably higher than that of liver in the same animal. The porphyrin-producing enzyme activity of the mouse Harderian gland extracts(2) varied with age of animal and the strain, but age of animal was the most important variable. The amount of porphyrin-producing enzyme appeared to increase rapidly just before mice became sexually mature, when the hypophysis increases its output of gonadotropic and perhaps other hormones. It was, therefore, of interest to examine the quantitative aspects of porphyrin-producing enzymes in Harderian glands of hypophysectomized rats to see if the gonadotropic hormones or secondarily produced sex hormones might have some influence on increasing rate of production of Harderian gland porphyrins. Enzyme activities of homogenates of liver tissues were also determined to serve as control tissue. Hypophysectomy in the male albino rat brought increased porphyrin-producing enzyme activity of the liver but not of the Harderian gland. Neither of these results had been anticipated.

Materials and methods. Harderian glands and liver tissues were obtained from adult normal and hypophysectomized male albino rats under ether anesthesia. Animals were housed in metal cages and given Purina Laboratory Chow and water *ad lib*. The method of Davidheiser and Figge(3) was used to determine quantity of porphyrin produced by extracts of Harderian gland and liver tissues from normal (control) and hypophysectomized rats. In brief, tissues assayed for enzyme activity were carefully weighed and ground with sand in buffered saline (pH 7.2). The sand and solid glandular homogenates were centrifuged to obtain aqueous extract of the homogenate. The clear aqueous extract was added to Klett tubes in varying amounts (0.1-1.6 cc). Buffered saline was added to bring volume of each tube to 1.6 cc. The substrate added to each tube was 1.4 cc of 0.01 M δ-aminolevulinic acid. After incubation at 37°C for 24 hours, concentrated 37% HCl was added to give a 25% HCl concentration. This stopped the reaction and cleared the solution so that the amount of porphyrin formed could be determined by comparison with standard porphyrin solutions in a Klett colorimeter. Protein content of each tissue was determined by Clark's method(4).

Results. The quantity of porphyrin produced by enzyme extracts of livers and Harderian glands from normal and hypophysectomized rats is shown in Table I. The amount of porphyrin produced by extracts of liver from the hypophysectomized rat is approximately twice that of liver extracts of normal animals. In the normal animal, the porphyrin-producing enzyme concentration of liver was less than that of the Harderian gland. However, in the hypophysectomized rat the reverse was noted. Protein concentration of each of these tissues was also determined. The protein content of livers of hypophysectomized animals was about 3% higher than in the control normal animal, probably because of a higher water content of livers of normal non-hypophysectomized animals. This dif-

TABLE I. Porphyrin Production by Aqueous Extracts of Homogenates of Harderian Gland and Liver Tissue from Normal and Hypophysectomized Rats.

Tissue	Type of rat	Enzyme extract added 0.4 cc	Enzyme extract added 1.6 cc
		mg of porphyrin/24 hr	
Liver	Normal (control)	28.0	70.4
	Hypophysectomized	55.6	149.6
Harderian gland	Normal (control)	29.8	100.4
	Hypophysectomized	30.0	100.0

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ference would not be large enough to account for the 100% increase in enzyme activity. Harderian gland extracts of normal and hypophysectomized animals exhibited no significant difference in amount of porphyrin produced.

Discussion. Since such a marked change in amount of porphyrin-producing enzyme activity of the Harderian gland at puberty had been observed, it was anticipated that there would be some corresponding influence of hypophysectomy on porphyrin production of extracts of Harderian glands, but this was not observed.

In addition to great increase in porphyrin production by the Harderian gland at puberty, other observations also indicated that there might be some relationship between the hypophysis and the function of other porphyrin-producing glands such as sebaceous glands. It has been mentioned that there appears to be a cytological and functional similarity between Harderian gland and sebaceous glands(5,6). Sebaceous glands in the adult are also porphyrin-producing glands. It is a striking fact, that the sebum of the pre-pubertal child contains little or no porphyrin as judged by fluorescence of the sebum. At puberty, the sebum in certain areas of the skin becomes red fluorescent because there is a gradual increase in amount of porphyrin which continues until the age of 25. Red fluorescence of the sebum then appears to level off and a constant intensity is observed until age 55 or 60 when there is a decrease in amount of porphyrin in sebaceous material. That the hypophysis influences sebaceous glands was shown by Lasher, Lorincz, and Rothman(7) who observed that there was atrophy of cutaneous sebaceous glands in ovariectomized and hypophysectomized white rats. It was also observed by Lorincz and Lancaster(8) that hypophysectomized castrated male rats were affected by injections of sebatropic preparations. It is well established that the hypophysis affects growth of the Harderian gland(9,10). It has been suggested that the growth hormone and thyrotropic hormone are responsible for such growth stimulation. It was therefore surprising to find that hypophysectomy appeared to have no influence on porphyrin-producing potentialities of the Harderian gland as determined by testing the extracts of homogenized tissues.

It was equally surprising to find that the amount of porphyrin-producing enzyme in liver extracts of hypophysectomized rats was double that of controls. The fact that liver tissues have such a great capacity to manufacture porphyrin indicates that rate of turnover of these substances may be relatively rapid. Why hypophysectomy should cause increased rate of porphyrin manufacture in the liver, however, is not understood.

Summary and conclusions. 1. A quantitative study was made of porphyrin-producing enzyme activities of Harderian glands and livers of normal and hypophysectomized albino rats. 2. Buffer-saline extracts were made from weighed quantities of each homogenized tissue. Measured amounts of extracts were mixed with 0.01 M solution of δ-aminolevulinic acid and incubated at 37° for 24 hours. 3. Extract of Harderian glands of normal and hypophysectomized rats showed approximately equal amounts of porphyrin-producing enzyme activity. The hypophysis appears to have little or no influence on this phase of activity of the Harderian gland. 4. Extracts of livers of hypophysectomized animals exhibited approximately twice as much porphyrin-producing enzyme activity as extracts of liver of normal animals. Extracts of liver from hypophysectomized rats also showed greater activity than extracts of Harderian glands. 5. It was concluded that the hypophysis exerts a relatively potent influence on porphyrin production by the liver.

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Effect of Ovariectomy and Replacement Therapy Upon Thyroxine Secretion Rate of Rat.* (25414)

RICHARD C. MOON† AND CHARLES W. TURNER
Dept. of Dairy Husbandry, University of Missouri, Columbia

The role of ovarian hormones in influencing thyroid function has been the subject of many investigations. It has been reported that ovariectomy depresses(1,2) or has no effect upon thyroid function(3,4). Moreover, estrogen administration may increase(5,6,7), decrease(8,9), or exert little effect(3) upon thyroid activity depending upon dose and length of treatment. Progesterone, on the other hand, increased thyroid function(5,10) but when administered concomitantly with estrogen, a depressing effect has been reported (11). In the above studies various indices of thyroid activity have been used, such as release of thyroidal-I^{131}, I^{131} uptake, thyroid histology and cytology and hyperplasia. Since there is little agreement concerning the problem, it seemed desirable to determine quantitatively the influence of ovariectomy upon thyroxine secretion rate (TSR) at various periods postcastration and effect of ovarian hormone replacement therapy.

Materials and methods. Young mature female rats of Sprague-Dawley-Rolfsmeyer strain with initial weight of 190-230 g were housed at uniform temperature of 78 ± 1°F in a room artificially illuminated during normal daylight hours. They were given Purina Lab Chow and fresh water daily. Each rat was injected i.p. with 2 μc carrier-free I^{131}. Forty-eight hours were allowed for I^{131} fixation by the thyroid. External neck counts were taken at this time and every 48 hours thereafter by first anesthetizing each animal with ether, then placing it on a lead plate with neck resting on a scintillation probe containing a 2″ NaI crystal. Care was taken in placement of animal to insure same geometrical relationship at each successive counting period. Thyroidal radioactivity was measured with scintillation counter, Nuclear-Chicago (N.C.) Model DS5, connected to rate meter, N.C. Model 1620 A. Conventional corrections were made for decay of isotope and background. *Determination of thyroxine secretion rate (TSR).* Each rat was injected subc. with .5 μg/100 g l-thyroxine for 2 consecutive days beginning 4 days after I^{131} injection, in such manner that ovariectomized rats received initial thyroxine injection either on day of ovariectomy or at days 6, 20, 70, and 84 postcastration. Thyroxine dose was increased at .5 μg/100 g increments, each dose injected for 2 consecutive days, with neck counts made on day of each increase. TSR was determined by plotting the percent previous count with thyroxine dose. The dose which prevented further thyroidal-I^{131} output in each rat (95-100% previous count) was estimated as its TSR. Some ovariectomized rats received daily subc. injections of 1 μg estradiol benzoate (EB) and/or 3 mg progesterone (P), commencing day of initial thyroxine injection and continuing until their TSR was established. Intact rats of same age and body weight range served as controls for each series of TSR determinations.

Results. Effect of ovariectomy and replacement with EB and/or P upon average daily thyroxine secretion rate (TSR) is pre-

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† Postdoctoral Fellow of N.I.H. This investigation supported by grants from P.H.S. and Am. Cancer Soc. Present address: Dept. of Physiology, Univ. of Tennessee, Memphis.

TABLE I. Effect of Ovariectomy and Estradiol Benzoate (EB) and/or Progesterone (P) Replacement upon Thyroxine Secretion Rate in Rats.

Treatment	No. of rats	Avg Body wt, g	Avg TSR, μg l-thyroxine/100 g/day	Probability
Series I				
Intact	18	217.4	1.08 ± .06‡	
Ovariectomized (2-6)*	16	223.0	.72 ± .06	.01
Series II				
Intact	41	239.9	1.09 ± .08	
Ovariectomized (8-12)	16	244.2	.63 ± .07	.01
" (22-28)	17	253.8	.70 ± .09	.02
Series III				
Intact	10	265.0	1.10 ± .16	
Ovariectomized (72-74)	11	304.1	.64 ± .10	.05
" (86-90)	13	308.9	.62 ± .06	.01
Series IV†				
Intact	12	244.5	1.20 ± .16	
Ovariectomized + 1 μg EB (2-8)	12	227.1	1.13 ± .11	
Idem + 3 mg P (2-8)	12	224.9	1.17 ± .09	
Ovariectomized + 3 mg P (2-6)	15	255.0	.73 ± .11	.02
Aggregate				
Intact	81		1.11 ± .05	
Ovariectomized	73		.66 ± .03	.001

* Denotes days ovariectomized before TSR was established.
† Initial EB and/or P inj. on day of ovariectomy.
‡ Stand. error of mean.

sented in Table I. TSR of ovariectomized rats determined immediately postcastration (2-6 days) was .72 μg/100 g l-thyroxine. This represented a decrease of approximately 33% when compared with intact controls of same age and body weight range. Increasing the period of ovariectomy to approximately 3 months did not produce any further significant lowering of TSR. Differences in TSR between respective age controls were also insignificant. Ovariectomized rats receiving 1 μg EB alone and with 3 mg P exhibited TSR's (1.13 and 1.17, respectively) in range of intact controls. Administration of 3 mg P alone did not significantly alter the decrease in TSR which resulted upon ovariectomy.

Discussion. Several investigators have attempted to define the relationship between ovarian and thyroid function. Contradictory results in previous studies(1,3,5,7) may be due to lack of sensitivity of methods employed for measuring thyroid activity. Our investigation, however, clearly indicates a reduction in thyroid function following ovariectomy since TSR was reduced approximately 33% as compared with intact controls. It also appears that reduction in thyroid function occurs in a relatively short period of time, since no further decrease in TSR was evident in rats ovariectomized 80 days or more. Although the mechanism by which ovariectomy reduced thyroid activity is not clear, the data from injection of 1 μg EB alone, and with 3 mg P appear to implicate a diminution in circulating estrogen level as a contributing factor since TSR of ovariectomized rats receiving estrogen was maintained at control level, whereas injection of P alone had little or no effect in maintaining TSR. It has been shown that a single injection of 1 μg EB increases 6 hour I^{131} uptake in ovariectomized rats, but has no effect in hypophysectomized-ovariectomized animals(5). This suggests that estrogen may stimulate increased secretion of thyrotropin (TSH) by adenohypophysis and a subsequent increase in I^{131} uptake by the thyroid. More recent studies(7, 12, 13) indicate large doses of estrogen, administered for prolonged periods of time, may stimulate thyroid directly. It is likely, however, that ovariectomy results in a lower circulating estrogen level and this, in turn, may

reduce TSH secretion and a subsequent reduction in thyroid activity. Such an interpretation appears consistent with observations that a physiological level of estrogen prevents reduction in TSR following ovariectomy and only increases thyroid activity if the adenohypophysis is intact(5).

Summary. 1) Effect of ovariectomy and replacement with 1 μg estradiol benzoate (EB) and/or 3 mg progesterone (P) upon daily thyroxine secretion rate (TSR) has been studied in mature rats. 2) Ovariectomy reduces TSR approximately 33% within 2-6 days postcastration. Increasing the interval to approximately 90 days between ovariectomy and TSR determination did not result in a further reduction in TSR. EB alone and with P prevented decrease in TSR following ovariectomy but P alone had no effect. 3) It is suggested that ovariectomy reduces the circulating estrogen level which, in turn, may reduce thyrotropin secretion and, subsequently, lower daily TSR.

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Effects of Age, Sex and Line on Serum Alkaline Phosphatase of the Chicken.* (25415)

YUICHI TANABE† AND F. H. WILCOX (Introduced by C. S. Shaffner)
Dept. of Poultry Husbandry, University of Maryland, College Park

Serum alkaline phosphatase has been studied to some extent in the chicken. Its level has been reported to be higher in the young chicken than in the adult(1,2), and higher in the laying hen than in the adult male(1,3,4). Gutowska *et al.*(5) reported serum alkaline phosphatase in high producing hens to be higher than that of poor producing ones. However, other workers(1,6) reported that it bears no relation to rate of egg production. Several workers(1,4,6) have observed no significant difference between the phosphatase values of laying and those of non-laying females. A high line for serum alkaline phosphatase has recently been developed by selection based on activity of the enzyme measured at 6 weeks of age in a random bred population of White Leghorn chickens (Wilcox and Shaffner, unpublished). The present study was conducted to determine to what extent, if any, the high line differs from its random bred control at other ages. This might in turn also provide information as to the physiological nature of genetic modification in the activity of this enzyme. The present work was undertaken also to elucidate the effects of age and sex on serum alkaline phosphatase in the chicken.

Materials and methods. Males and females of a high serum alkaline phosphatase line and a random control line of White Leghorn chickens were used. The birds were fed *ad libitum*. Blood was taken at different ages from 10 males and 10 females randomly selected from

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† Present address: Dept of Livestock, Nat. Inst. of Agricultural Sci., Chiba-Shi, Japan.

TABLE I. Effects of Age, Sex and Line on Serum Alkaline Phosphatase of the Chicken.

	High line				Random line			
	♂		♀		♂		♀	
Age, wk	Body wt, g	Alkaline phosphatase*	Body wt, g	Alkaline phosphatase	Body wt, g	Alkaline phosphatase	Body wt, g	Alkaline phosphatase
0	38	71.6 ± 9.3†	37	68.9 ± 2.0	36	69.4 ± 4.7	36	74.4 ± 3.2
1	60	437.5 ± 41.7	62	401.9 ± 42.4	60	398.4 ± 46.8	62	272.7 ± 34.5
2‡	119	589.4 ± 77.2	114	439.0 ± 83.9	118	350.2 ± 56.7	108	282.0 ± 44.8
3‡	194	364.0 ± 75.4	175	265.6 ± 31.6	204	274.2 ± 31.6	181	167.8 ± 15.1
4	263	158.8 ± 25.2	250	107.3 ± 19.0	281	133.6 ± 19.7	252	91.6 ± 17.8
6‡	480	105.4 ± 14.8	421	54.3 ± 10.8	463	40.7 ± 4.8	444	35.5 ± 6.3
8‡	682	117.2 ± 17.7	559	72.5 ± 10.3	639	73.7 ± 12.9	539	58.0 ± 10.2
10‡	954	60.1 ± 13.5	752	47.6 ± 7.6	985	38.0 ± 5.4	752	20.4 ± 2.0
14‡	1473	18.9 ± 1.6	1061	18.3 ± 2.0	1477	16.5 ± 1.3	1056	12.4 ± 1.7
18	1711	22.6 ± 2.3	1330	33.4 ± 3.6	1830	21.6 ± 1.4	1278	28.7 ± 4.1
22‡	1900	18.8 ± 1.5	1623	27.8 ± 3.0	2086	12.4 ± 1.1	1653	16.8 ± 2.2
26‡	2286	22.7 ± 1.5	1802	32.7 ± 2.3	2371	14.7 ± 1.3	1789	20.9 ± 3.3
30	2372	13.9 ± 1.9	1911	23.4 ± 2.7	2497	9.0 ± .7	1799	22.9 ± 2.4
34	2408	13.2 ± 2.6	1914	22.0 ± 1.4	2517	6.4 ± .6	1841	20.0 ± 1.9

* Expressed as mM of nitrophenol liberated/liter of serum/hr.
† Mean of 10 birds and stand. error.
‡ Indicates statistically significant line differences of combined data from males and females.

each line. At ages below 14 weeks it was taken by heart puncture from different sets of birds. After 14 weeks of age it was taken from the wing vein of the same 10 males and females. Serum alkaline phosphatase was determined by the method of Bessey *et al.*(7). Serum separated from blood of the chicken was incubated with the para-nitrophenyl phosphate substrate, pH of which was adjusted to 10.3-10.4, for 30 minutes at 37°C. The unit of alkaline phosphatase activity was expressed as mM of nitrophenol liberated per liter of serum per hour. Duplicate analyses were made on each blood sample.

Results. Results are summarized in Table I. Serum alkaline phosphatase level increased rapidly after hatching in both lines, until it reached a peak at 2 weeks of age. Thereafter in the male it continued to decrease and reached a low level in the adult, whereas in the female it decreased with advancing age until 14 weeks of age. At 18 weeks of age serum alkaline phosphatase of the female increased somewhat, but subsequently decreased gradually with advancing age. Values at 34 weeks of age represent only one-twentieth of peak value in the female, and one-fiftieth in the male.

No sex difference was observed at hatching time in either line. In the period of 1-14 weeks of age serum alkaline phosphatase was always numerically higher in the male than in the female. These differences are statistically significant at 6 and 8 weeks of age in a high line, and 1, 3 and 10 weeks of age in a random line. However, in the period of 18-34 weeks of age the difference between sexes was reversed, and alkaline phosphatase in the female was always numerically higher than that in the male. The differences are statistically significant at 30 and 34 weeks of age in both lines, and at 18, 22 and 26 weeks of age in a high line. This high value in the female appears to be related to egg production in this period, as most of the hens started egg production during 22-26 weeks of age, and all of the hens used here were laying at 30 and 34 weeks of age. The value in non-laying hens 35 weeks of age, which had started egg production but stopped the production, was low (6.5 ± 1.3 units, average of 4 birds), compared with 20-22 units in the laying hens at approximately the same age.

Although the variation of serum alkaline phosphatase with age was similar in both lines, the value for the high line was always numerically higher than that for the random bred line, except at hatching time. These differences were statistically significant in most cases from the age of 2 weeks to 26 weeks. No difference in body weight was observed between the 2 lines.

Changes in serum alkaline phosphatase level due to age correspond well with growth

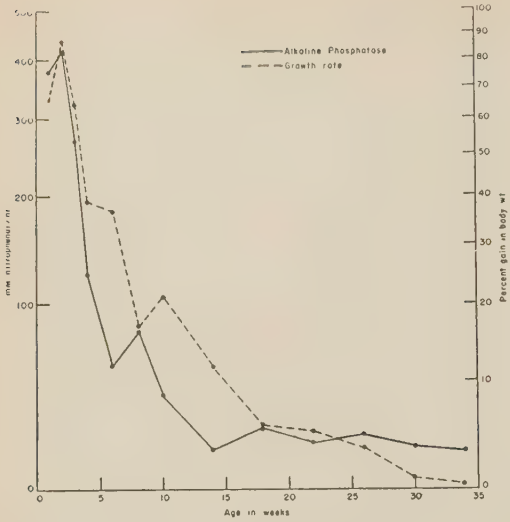

FIG. 1. Relation of serum alkaline phosphatase level of blood serum to growth rate expressed as % gain/wk.

rate expressed as percentage gain per week (Fig. 1). Both alkaline phosphatase level and percentage gain per week reached a peak at 2 weeks of age and decreased rapidly with advancing age.

Discussion. The present study demonstrates that serum alkaline phosphatase level is extremely high in the young chicken and reaches a low level in the adult. These changes correspond well with growth rate, and may be due to differences in bone formation, since this organ is known to be a source of serum alkaline phosphatase(9,10). The higher alkaline phosphatase activity usually observed in serum of the growing male in our experiments may be related to its faster growth. Serum alkaline phosphatase in the laying hen is significantly higher than that of the cock and non-laying hen as shown by the present work. This observation is in disagreement with the results of Common(1) and others(4,6) who found no significant difference between laying and non-laying hens. The difference between adult males and females is difficult to explain, since injection of estrogen decreases serum alkaline phosphatase in the chicken(8). It could possibly be due to a higher activity of the thyroid or to a higher demand for calcium for egg shell formation. It has been noted that calcium deficiency increases serum alkaline phosphatase in the chicken(1).

A numerical line difference was observed in all ages of the chicken except at hatching time. This indicates that in a line bred for a high level of alkaline phosphatase at 6 weeks of age the increased level is largely present at most other ages in the growing bird, and also in adult fowl.

Summary. Serum alkaline phosphatase was found to be higher in most cases between 2 and 26 weeks of age in a line bred for high alkaline phosphatase than in a random bred control line. The level in the male was higher than the female during the growing period, but lower at later ages. There were no differences due to line or sex at hatching.

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Amino Acid Requirements for Growth of Avian Lung Cultures.* (25416)

Robert E. Neuman and Alfred A. Tytell
Virus and Tissue Culture Research Division, Merck Inst. for Therapeutic Research, West Point, Pa.

Amino acid requirements for growth of a large number of stable line mammalian cells (1) primary rhesus monkey testicular† and primary monkey kidney(2) cell cultures have been determined. Morgan and Morton reported the effects of amino acids upon prolonging survival of chick embryo heart cultures in synthetic media(3). However, the amino acids essential for growth of primary avian cultures have not been reported. The present paper describes amino acid requirements for growth of primary chick embryo lung and cockerel lung cultures.

Methods and materials. Cell suspensions of 14-16 day White Leghorn chick embryo lungs and 2-6 week old cockerel lungs were prepared by stirring 2-4 hours with 0.2% trypsin (Bacto-Difco 1:250) in Earle's balanced salt solution at 37°C. The cell suspension was filtered through gauze, collected by centrifugation and resuspended in a complete medium. One ml amounts of a suspension containing 50,000-100,000 cells per ml were pipetted into 15 x 125 mm tubes which were placed in a slanted position in racks. The cultures were established by incubation for 24 hours at 37°C in an atmosphere of 8% carbon dioxide-92% air The medium was removed and the tubes were rinsed with Earle's solution. The cells then were overlaid with 1 ml of test medium containing graduated amounts of the test amino acid. Incubation was continued for 3-5 days until the cells formed a sheet or attained maximal growth in presence of optimal amounts of the test compound. The medium was not replenished. Growth was measured by the protein determination method of Oyama and Eagle (4). The complete medium in which the cells were established was similar to that previously described(5). It contained l-isomers of the amino acids as well as 0.2 mM pyruvate, 10 μg adenosine per ml, 5% bovine serum, and 6% chick embryo extract. The test medium differed in that it contained dialyzed serum and dialyzed embryo extract. Adenosine and glutathione were omitted. Embryo extract was prepared from 12 day chick embryos ejected through the orifice of a 20 ml B-D Luer-Lok syringe. The brei was mixed with an equal volume of Earle's solution and after 1 hour the supernate was collected by centrifugation. Expressions of quantity of embryo extract were based on this preparation. However, this material was diluted with 2-3 volumes of Earle's solution prior to sterilization by filtration through Selas filter of 02 porosity. Dialysis of serum and embryo extract was conducted as described previously(5). Earle's solution was modified in these studies to contain 3.0 g glucose and 6.46 sodium chloride per liter.

Results. Initial investigation demonstrated the requirements of both chick embryo lung and maturing cockerel cultures for the non-dialyzable moieties of embryo extract and serum. In Fig. 1 are shown typical growth responses to dialyzed chick embryo extract. In Fig. 2 are shown the typical growth responses to dialyzed pooled bovine and chick serum. The bovine serum was superior to the chick serum. The presence of both dialyzed embryo extract and serum was essential for growth of the cultures in the test medium. High concentration of either embryo extract or of serum did not eliminate the requirement for the other component. For the amino acid studies, dialyzed embryo extract and dialyzed serum were included in the medium at 6 and 5%, respectively, in order to minimize possible contributions of amino acids by these materials.

By varying the amount of each amino acid in turn over a concentration range of 0-1.0 mM, the 13 amino acids usually required by

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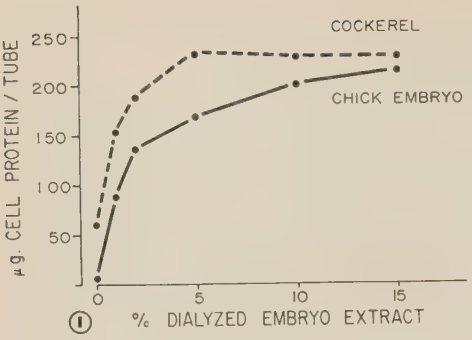
† Described in abstract: Tytell, A. A., Rader, Y., Krumm, D. L., *Fed. Proc.*, 1958, v17, 326.



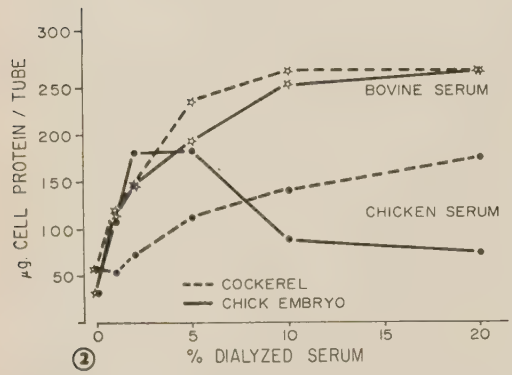


FIG. 1. Growth response of primary chick embryo and cockerel lung cultures to dialyzed embryo extract. Medium contained 5% dialyzed bovine serum.

FIG. 2. Growth response of primary chick embryo and cockerel lung cultures to dialyzed chick and bovine serum. Medium contained 6% dialyzed embryo extract.

mammalian cells(1) were found to be essential for chick embryo lung cultures. The results are presented in Table I. The optimal levels of these amino acids were similar to those for mammalian cells. Microscopic examination of cultures in the absence of any one of the essential amino acids revealed cellular deterioration. Glutamic acid up to levels of 50 mM only partially substituted for glutamine. The cockerel lung cultures were found to have amino acid requirements identical with those of the chick embryo cultures.

A study of the other amino acids and derivatives in the test medium demonstrated the stimulatory effects of glycine, L-serine, L-alanine, and pyruvate. Growth response of chick embryo lung cultures is shown in Table II. Addition of 0.1 mM of either glycine or L-serine to the test medium from which both these amino acids had been omitted resulted in marked stimulation. Furthermore, inclusion of 0.1 μg folinic acid or 5-10 μg folic acid per ml (the test medium contained 0.2 μg folic acid per ml) stimulated the cultures in the absence of added glycine or L-serine, although maximal growth was not attained. Addition of either 0.01 mM L-alanine or 0.1 mM pyruvate to the test medium from which L-alanine and keto acids had been omitted resulted in stimulation. Oxalacetate or alpha-ketoglutarate at 0.2 mM also replaced alanine. Lactate, isocitrate, citrate, cis-aconitate, malate, succinate, fumarate, glutamate, aspartate, folinic acid, and higher levels of folic acid, pyridoxine and pyridoxal did not substitute for L-alanine. Pyridoxal phosphate (1-10 μg/ml) did stimulate the chick lung cells to a limited extent in the absence of L-alanine. Thus a medium containing the 13 essential amino acids also required glycine, L-alanine or related compounds to attain growth comparable to that on the original test medium which contained 20 amino acids. Other "non-essential" amino acids were omitted from the medium without affecting growth. Primary cockerel lung cells responded to glycine, L-alanine, and

TABLE I. Amino Acid Requirements for Growth of Primary Chick Embryo Lung Cultures.

	Concentration of amino acid (mM)							
Amino acid	0	.01	.02	.05	.1	.2	.5	1.0
	Growth response (inoculum = 1)*							
L-arginine	1.5	4.0	6.3	8.6	9.5	9.8	9.3	9.3
isoleucine	1.7	3.1	6.0	10.0	12.6	12.3	12.7	14.0
leucine	2.3	4.6	6.0	9.7	14.4	15.7	15.4	11.3
lysine	2.9	4.9	7.0	9.9	14.0	14.6	14.7	14.7
methionine	1.5	3.3	5.8	7.0	7.4	7.5	7.3	5.2
phenylalanine	2.5	4.0	6.4	6.6	7.0	7.3	7.8	6.1
threonine	1.7	3.2	4.2	6.3	7.6	7.2	5.2	4.6
tyrosine	3.4	6.6	11.0	14.4	14.7	15.0	14.6	13.1
valine	.8	2.9	7.3	7.0	7.9	8.9	8.3	8.3

	Concentration of amino acid (mM)						
	0	.1	.5	1.0	1.5	2.0	5.0
L-glutamine	1.7	4.3	11.0	13.3	14.7	14.6	15.6

	Concentration of amino acid (mM)							
	0	.001	.002	.01	.02	.05	.1	.2
L-cysteine	1.6	2.6	3.3	7.9	10.9	14.0	14.3	13.1
histidine	2.4	3.7	4.1	7.6	12.3	14.7	14.4	14.7
tryptophane	1.9	2.9	4.9	14.3	15.5	16.4	16.0	14.7

* Inoculum refers to 24 hr plated cells subjected to the test medium.

TABLE II. Stimulatory Effects of Glycine, L-serine, L-alanine, and Pyruvate on Primary Chick Embryo Lung Cultures.

Compound	Concentration (mM)									
	0	.001	.005	.01	.02	.05	.1	.2	.5	1.0
	Growth response (inoculum = 1)*									
Glycine†	9.4			10.4	11.4	12.6	13.0	12.4	11.4	11.2
L-serine†	9.4			11.2	11.8	12.6	15.0	15.2	17.2	16.8
L-alanine‡	8.3	9.8	12.3	15.1	15.0	15.1	15.5	14.5		
Pyruvate‡	8.3			10.9	12.0	13.0	14.1	14.1	13.4	13.4

* Inoculum refers to 24 hr plated cells subjected to test medium.

† Test medium contained 13 essential amino acids plus L-alanine, pyruvate, L-asparagine, L-aspartate, L-glutamate, L-proline, L-hydroxyproline.

‡ Test medium contained 13 essential amino acids plus glycine, L-serine, L-asparagine, L-aspartate, L-glutamate, L-proline, L-hydroxyproline.

related compounds in the same manner as did the embryonic cells.

Discussion. A major difference in the nutritional requirements of avian and mammalian cultures which has received detailed study appears to be a factor or factors associated with the macromolecular constituents of embryo extract(6,7). Additional factor(s) associated with the nondialyzable portion of serum are required by both groups of cells. The serum factor(s) were not species specific. In fact, bovine serum was more effective than chick serum and did not inhibit at higher levels as did chick serum upon occasion. The requirement for embryo extract was not confined to embryonic tissues but applied as well to the maturing cockerel cells. However, these studies were over brief growth periods on a gross population of primary cells. Adaptation or stabilization processes in continued culture could result in strains with different properties. At present primary chick embryo lung cultures have proved highly satisfactory in this laboratory in assay procedures in studies of embryo extract growth factor(s). Kutsky has prepared growth-promoting proteins from tissue extracts which were assayed using explants or subcultured cells(6,7).

Avian cells in common with mammalian cells exhibited requirements for 13 essential amino acids. Avian cells also were stimulated by glycine, L-serine and to a lesser degree by 0.1 μg folinic acid or 5-10 μg per ml of folic acid. These stimulatory effects of glycine, L-serine, folinic acid and higher concentrations of folic acid frequently have been encountered in this laboratory in a wide variety of mammalian cultures. Substitution of glycine by folinic acid for growth of primary monkey kidney cultures has been reported (8).

An outstanding difference in the micromolecular requirements for optimal growth of avian cultures was the marked stimulation by small quantities of L-alanine. Relatively high levels of pyruvate, oxalacetate, alpha-ketoglutarate could replace the alanine requirement perhaps by contribution to its biosynthesis. Pyridoxal phosphate improved growth in some measure in the absence of L-alanine although the test medium contained a total of 1 μg per ml of pyridoxine and pyridoxal. The participation of this cofactor in transamination reactions in production of L-alanine(9) may account for its activity. Isolated Walker carcinosarcoma 256 cells have been shown to require pyruvate, oxalacetate, or alpha-ketoglutarate, although alanine could not substitute for these compounds(5).

Summary. Primary chick embryo and maturing cockerel lung cultures were found to grow in a test medium containing dialyzed chick embryo extract, bovine serum, and a variety of small molecular constituents. The avian cultures required the 13 amino acids usually found to be essential for mammalian cells. They also were markedly stimulated by glycine or L-serine and by small quantities of L-alanine. Folinic acid and higher levels of folic acid partially replaced the glycine requirement for optimal growth. Pyruvate, oxalacetate, or alpha-ketoglutarate, replaced the requirement for L-alanine. Pyridoxal phosphate partially replaced the L-alanine.

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Rectal Absorption of 6-Alpha-C^{14}-H_3-Prednisolone.* (25417)

JEAN A. SPENCER, JOSEPH B. KIRSNER AND WALTER L. PALMER
Dept. of Medicine, University of Chicago

Administration of adrenocortical steroids per rectum in treatment of ulcerative colitis has produced encouraging results(1-11). In general, the primary mode of action of this form of therapy has been attributed to a local effect, as in the topical treatment of certain dermatologic conditions(2,5,6,10,14) though Liddle(13) and Nabarro *et al.*(4) have demonstrated absorption of some forms of hydrocortisone given by rectum. Our first experience with rectal administration of steroids was in Feb., 1958 during a preliminary evaluation of 6-methyl-prednisolone. The response was surprisingly gratifying. The subsequent availability of radioactive 6-methyl-prednisolone (6-alpha-C^{14}-H_3-prednisolone) prompted studies to (1) estimate amount of steroid absorbed from the rectum, (2) determine the rapidity with which it might be absorbed, and (3) length of time it remained within the body, if absorbed.

Methods. Administration: One milligram (3.2 microcuries) of 6-alpha-C^{14}-H_3-prednisolone, dissolved in one ml of 95% ethanol, was drawn into a 20 ml syringe. Forty mg of 6-methyl-prednisolone sodium succinate, dissolved in 1 ml of water, were rapidly drawn into the same syringe; (in case 4 this step was eliminated). Fifteen milliliters of tap water were drawn into the same syringe. The contents of the syringe were mixed rapidly and emptied by gravity into a soft rubber catheter, whose tip had been placed in the rectum of the patient lying on his left side. The catheter drained into the rectum completely within one minute and then was withdrawn; no patient evacuated the rectum in the succeeding 24 hours. *Analysis of urine*: Two successive 24 hour urine collections were made as separate voided specimens. The volume and time of each voided urine were recorded. Aliquots (0.5 ml) from each specimen were counted in a Packard Tri Carb Scintillation Counter as disintegrations per minute; total disintegrations/minute per voided specimen were calculated from these data. All voided specimens for each 24 hour period were pooled and mixed. Nine hundred ml aliquots from each 24 hour pool were extracted for the neutral lipid fraction. *Extraction of urinary corticosteroids*:† (12) The urinary pH was adjusted to 5. One-tenth volume of acetate buffer (pH 5.0) was added. Hydrolysis with 300 units of spleen beta-glucuronidase/ml progressed 5 days. The hydrolyzed urine was adjusted to pH 1 with sulfuric acid and continuously extracted with ether for 48 hours. The ether extract was washed 3 times with each 1/10 volume 2N sodium hydroxide and 1/10 volume of 5% sodium chloride. Each hydroxide or salt washing was back extracted with ether. Each "back extract" was added to the original ether extract. The total ether extract was evapo-

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† Modification of the method of Dobriner made by Attallah Kappas—personal communication.

TABLE I. Rectal Absorption of Radioactive 6-Methyl Prednisolone.

	Patient 1	Patient 2	Patient 3	Patient 4
Rectal dose (3.2 μc)				
Total counts/ml	10,870,000 DPM	10,870,000 DPM	10,870,000 DPM	10,870,000 DPM
Urine				
(1st 24 hr)	3,811,390	3,270,090	1,704,800	6,583,380
(2nd " ")	182,610	229,560	275,416	372,190
Total urinary radioactivity	3,994,000 DPM	3,499,650 DPM	1,980,216 DPM	6,955,570 DPM
% of rectal dose	37%	31%	18%	64%
Urinary ethereal extract				
(1st 24 hr)	2,111,787	1,297,399	993,378	3,338,048
(2nd " ")	90,372	Not done	153,868	157,016
Total	2,202,159 DPM		1,147,246 DPM	3,495,064 DPM
% of urinary radioactivity	55%		57%	50%
Urinary neutral lipid extract				
(1st 24 hr)	583,204	402,185	355,374	900,176
(2nd " ")	67,648	6,399	126,902	94,480
Total	650,852 DPM	408,584 DPM	482,276 DPM	994,656 DPM
% of urinary radioactivity	16%	12%	24%	14%
Residual urine				
(1st 24 hr)	1,197,990	892,080	326,981	40,493
(2nd " ")	97,640	Insufficient count	31,907	2,070
Total	1,295,630 DPM		358,888 DPM	42,563 DPM
% of urinary radioactivity	32%	25%	18%	0.6%

DPM = Disintegrations/min.

Patient 1 (S.R.)—Normal. Patient 2 (M.F.)—Chronic ulcerative colitis under no therapy. Patient 3 (R.H.)—Chronic ulcerative colitis receiving prednisone, 40 mg daily. Patient 4 (I.B.)—Chronic ulcerative colitis receiving ACTH gel, 40 units intramusc., and prednisolone, 100 mg orally daily.

rated to dryness, then dissolved in 25 ml of purified ethanol. The ether extract, sodium hydroxide washing and sodium chloride washing were counted in a Packard Tri Carb Scintillation Counter.

Results. Unfortunately, studies in patients with ulcerative colitis involving urine and fecal collections are fraught with difficulties indigenous to the disease. Of 7 hospitalized patients in whom the radioactive 6-methyl-prednisolone was instilled, only 2 ulcerative colitis patients and one normal individual provided complete collections of urine. Athough a third ulcerative colitis patient (R.H.) maintains he supplied a complete collection, the low 24 hour volumes and low total radioactivity of his urine strongly suggest that the collections were not complete; nevertheless, this case is included.

The 4 patients in this study are: (1) S.R., a normal male 54 years of age; (2) M.F., a 25 year old male with chronic ulcerative colitis for 4 years who had not received steroid or corticotropin therapy for 16 months; (3) R. H., a 20 year old male with ileocolitis for 2 years and receiving prednisone, 40 mg orally at time of study; (4) I. B., a 34 year old male with ulcerative colitis for 5 years and receiving ACTH gel, 40 units intramuscularly and prednisolone, 100 mg orally each day during this study. All ulcerative colitis patients were under relatively similar control; averaging one bowel movement every one to 2 days. The stools of M.F. contained gross blood; the others were positive for occult blood (benzidine test). The rectal mucosa of M.F. was edematous, granular and slightly friable. In the other patients with ulcerative colitis, the rectal mucosa was edematous and granular but not friable. In spite of the limited number of patients, the preliminary findings appear of interest.

The pattern of excretion of radioactivity is depicted in Table I. The most striking observation is the general similarity of the curves of all patients. It also is apparent that half of the dose of total absorbed radioactive 6-methyl-prednisolone reflected in urinary ex-

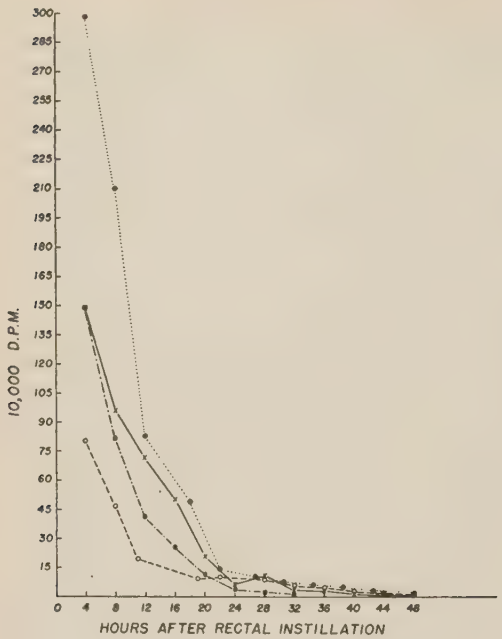

FIG. 1. Rectal absorption of 6-alpha-C^{14}-H_3-prednisolone. ×———×, Patient 1 (S. R.), normal; ■-·-·■, Patient 2 (M. F.), chronic ulcerative colitis with no therapy; ○– – –○, Patient 3 (R.H.), chronic ulcerative colitis receiving prednisone, 40 mg daily; ● ···· ●, Patient 4 (I. B.), chronic ulcerative colitis receiving ACTH gel, 40 units, and prednisolone, 100 mg daily.

cretion is excreted within 6 hours after rectal instillation, a trend observed in all 4 subjects. Two patients received tracer doses in the morning, 2 in the afternoon, there was no discernible diurnal variation in excretion.

Table II demonstrates amount of radioactivity from the rectal dose recovered in the urine. In the urine of the normal man 37% of the radioactivity in the rectal dose was recovered within 48 hours after instillation; 31% from the urine of the patient with ulcerative colitis receiving no other therapy. H.R., the patient receiving oral prednisone, excreted only 18% of the rectal dose in the urine, but, as stated previously, we suspect this low value reflects incomplete collection of urine, rather than a true decrease in absorption. I.B., the patient receiving ACTH intramuscularly and prednisolone orally, excreted 64% of the rectal dose in the urine. The increase in total absorption in this case may reflect an action of ACTH or prednisolone on the rectal mucosa, possibly increasing the absorptive capacity.

However, regardless of total amount of radioactivity excreted in urine, the various extractions of urine used to isolate the metabolites of 6-methyl-prednisolone contained approximately the same proportions of radioactivity in all cases. Between 50 and 75% of urinary radioactivity was contained within the metabolites extractable by ether. Between 12 and 24% was detected in the metabolites contained within the neutral lipid extract. This observation indicates that the major portion of steroid metabolites of 6-methyl-prednisolone are water soluble; an acceptable observation in relation to the known information concerning the metabolic products of prednisolone.

Discussion. Because the C^{14} atom is contained within the 6-methyl group, we have been concerned that bacterial action in the colon might split it off, permitting its absorption independently of the steroid nucleus. This may have occurred to the major portion of the 6-methyl-prednisolone instilled in the rectum. Nevertheless, the evidence indicates that a significant amount was absorbed unaltered. This result agrees with the findings of Nabarro *et al.*(4) and Liddle(5) who estimated that between 26 and 33% of hydrocortisone in alcohol was absorbed from the rectum. That plasma cortisol levels do not rise significantly following cortisol enemas(9) is not in disagreement with these findings. On the contrary, Schwartz *et al.* found that, although the plasma cortisol level was not altered measurably, the plasma content of conjugated steroids derived from cortisol was noticeably increased within the first hour after cortisol was given by rectum. These findings thus reflect the rapid metabolic turnover of adrenocortical steroids within the body.

The present observation that half of absorbed radioactivity excreted in urine leaves the body within 6 hours supports another observation of Schwartz *et al.*, that the elevated plasma content of conjugated steroids derived from radioactive cortisol given by rectum fell to non-detectable levels within 4 hours after the retention enema.

Although we do not know how much of the benefit of steroid retention enemas in treatment of ulcerative colitis is from local effect and how much from systemic absorption of the steroid, there is no doubt that a substantial amount of 6-methyl-prednisolone is absorbed. Evidence suggests that this drug is absorbed very rapidly, metabolized rapidly, and that half of the quantity absorbed and excreted in the urine is excreted in less than 6 hours.

Conclusion. 6-methyl-prednisolone is absorbed rapidly and in substantial amounts from the rectum in normal individuals and in patients with ulcerative colitis. Half of the 6-methyl-prednisolone absorbed from the rectum and excreted in the urine is eliminated within 6 hours. The beneficial action of 6-methyl-prednisolone retention enemas may be systemic as well as local.

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Action of Prolactin on Seminal Vesicles of Guinea Pig.* (25418)

HAROLD R. ANTLIFF, M. R. N. PRASAD† AND ROLAND K. MEYER‡
Depts. of Anatomy, Medical School and Zoology, University of Wisconsin, Madison

The function of prolactin (lactogenic hormone, luteotrophic hormone or LTH) in the male mammal is not well established. Pasqualini(1) reported a marked increase in secretions of seminal vesicles of castrate rats after administration of LTH for 3 consecutive days following priming dose of testosterone propionate. Influence of LTH on the prostate was not significant. Pasqualini considered that the action of LTH was directly on the seminal vesicles but manifested itself only when the wall of seminal vesicles was rendered reactive by action of testosterone. Chase *et al.*(2) observed minimal growth of seminal vesicles of castrate Sprague-Dawley rats with administration of LTH or LTH in combination with TP. Testosterone and STH together caused a significant increase in weight and proportion of glandular tissue of the ventral prostate and seminal vesicles. Their data suggest that LTH may act in a synergistic manner to stimulate accessories of the male rat. Everett(3,4) showed that pituitary autografts to the kidney capsule of female rats favored continuous secretion of LTH and maintained corpora lutea in a functional state at least 4 to 5 times the duration of pregnancy. Rennels (Univ. of Texas, personal communication) pointed out that the pituitary of male rat transplanted to kidney capsule of female rat was equally capable of producing LTH and maintained corpa lutea in a functional state. In view of these observations it became of interest to see if the autografted pitui-

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† Present address: Dept. of Zoology, University of Delhi, India.

‡ Authors acknowledge technical assistance of R. Anne Heian.

TABLE I. Effect of Prolactin (LTH) and Testosterone Propionate (TP) on Seminal Vesicles of Intact, Castrate, Hypophysectomized and Pituitary Autografted Male Guinea Pigs.

Group	Treatment TP (μg)	Treatment LTH (I.U.)	No. of animals	Body wt (g) Initial	Body wt (g) Final	Sem. ves. wt (g)	Epithelial ht ($m\mu$)
			Long term series				
Intact control			6	507	777	1873	17.8
		15	6	503	770	2036	18.0
Castrate control			3	510	715	352	8.1
		"	3	520	714	314	8.0
	12.5	"	3	530	670	490	8.0
	"	30	3	557	777	739	11.5
	"		3	517	705	364	8.8
Hypophysectomized			3	460	710	218	8.0
	"	15	3	510	805	326	9.0
	"	30	3	525	655	510	12.9
Pituitary autograft			3	490	564	222	7.8
	"		3	540	779	591	13.2
	"		3	530	640	478	8.5
			Short term series				
Castrate control			3	510	690	356	8.4
		30	3	531	583	363	8.7
*	25		3	573	580	879	17.8
*	"	30	3	575	616	870	17.7
*	"	60	3	530	760	1331	18.2
Intact control			3	515	744	2048	18.0

* Inj. with priming dose of 100 γ of TP for 6 days, followed by treatment indicated in Table for 7 days.

tary in the male guinea pig would function likewise. Experiments here reported are a pilot study, because dose levels of LTH in the guinea pig had to be established and combinations of LTH and TP relative to physiological conditions had to be checked. The consequence of this is evident in the design of the experiment in that many groups are involved, each containing only a few animals.

Materials and methods. Sixty-three male guinea pigs of a heterogeneous strain were used in 2 series of experiments, a long and short term series. Forty-five animals in the long term series were subjected to different operative procedures and placed in Groups 1 to 13 (Table I). All operations were performed when animals were 65 days old. Hypophysectomy was accomplished by parapharyngeal approach under ether anesthesia and when the gland was to be transplanted it was sucked into sterile cannula and placed in sterile physiological saline at room temperature. Autografts were made immediately by placing the gland under the left kidney capsule following the method of Everett(4). In Group 13, castration was done the same day immediately following autograft of pituitary gland. Daily intramuscular injections of testosterone propionate or prolactin were begun the day following surgery and continued for 8 weeks.§‖ Prolactin (NIH-SP-1) used is a highly purified sheep preparation, estimated to contain 20 I.U./mg, and distributed by the Nat. Insts. of Health. Pyrogen-free water at pH 7.0 was the solvent for the prolactin powder. On day following last injection, the animals were sacrificed by decapitation and sex accessories, pituitary, adrenals and testes were removed and weighed. Seminal vesicles were not freed of their contents before weighing. Bits of tissue from all these structures were fixed in Zenker's fluid, sectioned at 7 μ and stained with Groat's tetrachrome stain. The sella turcica of hypophysectomized animals was examined under magnification for presence of pituitary fragments. In the short term series, 18 male guinea pigs were castrated at 65 days of age. Six weeks

§ Prolactin was a gift from Endocrinology Study Section, Nat. Insts. of Health.

‖ Testosterone propionate was a gift from Ciba Pharmaceutical Products, Summit, N. J.

later, when sex accessories had attained maximum regression, they were divided into 5 groups and treated as indicated in Table I. Group 14 received no treatment. Groups 16, 17 and 18 were injected for 6 days with 100 μg of testosterone propionate/day after which they were injected for 7 days with 25 μg of testosterone propionate. In addition to above treatment, Groups 17 and 18 received 30 and 60 I.U. of LTH daily, respectively during last 7 days. Group 15 received only 30 I.U. of LTH daily during last 7 days without prior priming with TP. Animals were sacrificed the day following last injection and processed as in the long term series.

Results. In the long term series (Table I), castrates (Group 3) showed atrophy of seminal vesicles. The same condition was also seen in castrates treated with 15 I.U. LTH (Group 4), subminimal TP (12.5 μg) and 15 I.U. LTH (Group 5) or subminimal TP only (Group 7). Appearance of epithelium and average cell height in these groups was nearly the same (8.1 to 8.8 mμ). The nuclei were located basally and supranuclear zone was small. However, castrates treated with subminimal TP and 30 I.U. LTH (Group 6), exhibited seminal vesicles nearly double the size and weight of other treated groups (Groups 4, 5 and 7). Average cell height of epithelium was 11.5 mμ. Epithelium was slightly columnar, nuclei basal and supranuclear zone was tall and had a number of secretory granules in the cytoplasm. There was no visible accumulation of secretion in the seminal vesicles. Hypophysectomized animals receiving no treatment (Group 8) or those receiving subminimal TP and 30 I.U. LTH (Group 10) exhibited marked heightening of epithelium but with no visible secretion in the seminal vesicles. Since amount of secretion in seminal vesicles of different groups was too small for quantitative estimation, only a subjective evaluation was possible. In Groups 6 and 10 all of the seminal vesicles exhibited the responses indicated, with much uniformity present at the cellular level.

Pituitary autografted animals without further treatment (Group 11) showed marked atrophy of seminal vesicles corresponding to those of castrates, while those receiving subminimal TP (Group 12) evidenced an increase in weight of seminal vesicles and a cell height average of 13.2 mμ. Autografted castrates receiving same level of TP (Group 13) did not show any increase in epithelial height and resembled those of castrates.

The object of the short term series (Table I) was to verify and to test results of Pasqualini and Chase and check on a higher dose of LTH. In those treated with 30 I.U. LTH daily (Group 15) weight of seminal vesicles and appearance of epithelium were similar to castrates. Those treated with TP only (Group 16) had seminal vesicles weighing nearly double that of castrates and the epithelium was considerably higher (average cell height of epithelium 17.8 mμ). In animals treated with TP and 30 I.U. LTH daily (Group 17), weight of seminal vesicles was nearly the same as in those treated with TP only (Group 16). Appearance of epithelium was more like that of intact animals than androgen treated castrates. Nuclei were located basally in a row and the supranuclear zone of cytoplasm was tall and granular. A significant difference was noticed in Group 18 treated with TP and 60 I.U. LTH daily where weight of seminal vesicles was approximately twice that of groups treated with TP (Group 16) or those treated with TP and 30 I.U. LTH (Group 17). Average cell height of epithelium was the same as that of intact controls or those treated with TP only (17.8 mμ). All of the seminal vesicles of Groups 16, 17, 18 responded uniformly.

In neither series did the prostates, adrenals, pituitaries, testes and mammary glands show any significant differences in structure or size in the different groups treated with various levels of LTH.

Discussion. In long and short term series it appeared that seminal vesicles only, among sex accessories, were sensitive to LTH administration. While LTH alone had no effect on seminal vesicles of the castrate guinea pig, it did however cause a significant increase in weight and stimulated the epithelium of seminal vesicles primed with TP. Comparison of histological appearance of seminal vesicles in Group 6 with Group 17 of the short term series indicated that the epithelium was stimu-

lated. Presence of secretion in seminal vesicles of Group 17 only, indicated that higher priming dose of TP was required before LTH could cause it to become secretory. Doubling the dose of LTH to 60 I.U. daily (Group 18) caused corresponding increase in weight of seminal vesicles. Since there is no increase in height of epithelium the increase in weight could be accounted for primarily by increase in amount of secretion or by hyperplasia caused by LTH.

The difference in appearance of seminal vesicle epithelium between autografted intacts (Group 12) and autografted castrates (Group 13) may indicate that there was a small amount of secretion of gonadotrophic hormones by transplanted pituitary which stimulated the Leydig cells to produce testosterone. This augmented the subminimal TP injected exogenously. It is also possible that the epithelial response by autografted intact animals may indicate that small amounts of LTH were being produced by the graft. This acted synergistically with subminimal TP to bring about responses similar to those seen in animals receiving subminimal TP and a high dose of LTH.

When comparing groups 16 and 17, there seemed to be no significant change in seminal vesicle morphology resulting from daily administration of 30 I.U. LTH over that resulting from TP itself. Contrasting these 2 groups with Group 18 which received 60 I.U. LTH, it appeared as if some stimulation was obtained other than by TP. Thirty I.U. LTH acting with subminimal dose of TP in the long term study groups did show stimulation. It is clear that many levels of LTH must be tested, however, some quantitative relationships of effectiveness have been suggested.

Summary and conclusions. The role of LTH (luteotrophic hormone, prolactin or lactogenic hormone) in stimulating epithelium of seminal vesicles of castrated guinea pigs has been tested in 2 series of experiments. LTH alone had no effect on seminal vesicles of castrates. LTH together with subminimal testosterone propionate caused significant increase in weight of seminal vesicles as well as height of epithelium. A high priming dose of TP was required before LTH stimulated visible secretion. Doubling the dose of LTH after priming with TP caused a corresponding increase in seminal vesicle weight. Prostates, testes, mammary glands, adrenals and pituitaries did not show any significant differences in structure or size when various levels of LTH were present. The results are in agreement with the findings of Pasqualini(1) and Chase *et al.*(2).

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Distribution of Red Blood Cells Between Tissues of Mouse.* (25419)

J. J. FRIEDMAN† (Introduced by E. E. Selkurt)
Biophysics Laboratory, Tulane University, New Orleans, La.

Red blood cells labeled with radioactive isotopes are presently being employed in a variety of experimental conditions for determination of circulating red blood cell volume. In general, the technic consists of intravenous injection of labeled erythrocytes followed by sampling of blood after a period of time considered adequate for complete vascular mixing. Usually, this time interval is derived from the blood radioactivity curve and represents the point at which blood radioactivity achieves a constant level. While this interval may be suitable for determination of the cir-

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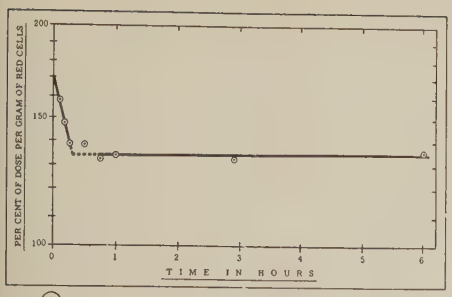
† Present address: Dept of Physiology, Indiana Univ. School of Med., Indianapolis.



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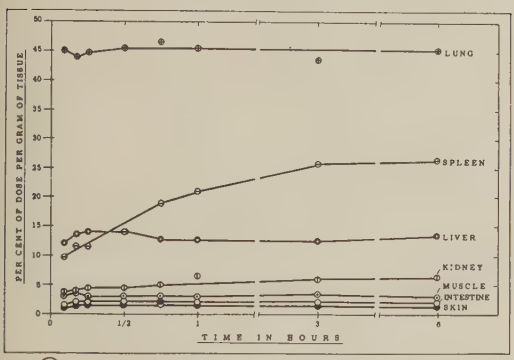

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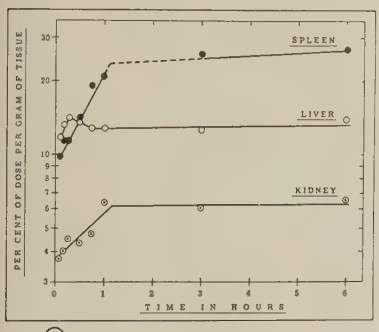

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FIG. 1. Concentration of radioactivity in circulation following intrav. administration of radioiron labeled red cells (semi-log plot).

FIG. 2. Distribution of intravenously inj. radioiron labeled red cells between various tissues of mouse.

FIG. 3. Distribution of radioactive red cells between liver, kidney and spleen following intrav. administration (semi-log plot).

culating red cell volume it does not necessarily indicate that vascular mixing has been completed at the tissue level. It is certainly conceivable that incomplete vascular mixing may exist in some segment of tissue vasculature and be obscured by the large pool of radioactivity in the effective circulation. Because it was of interest to study tissue circulation, a knowledge of the characteristics of local vascular mixing was considered essential. Therefore, the progressive accumulation of radioactivity in various tissues following intravenous injection of labeled red cells was determined.

Methods. Ninety male albino mice of the CF-1 strain weighing 19-22 g were used. Mouse red cells were labeled *in vivo* with radioactive iron (Fe-59) according to the method of Hahn *et al.*(1). Essentially the procedure consisted of intravenous injection of a donor animal with radioactive ferrous ammonium citrate, followed by removal of blood from donor animal at approximately 3 weeks post injection. Three weeks of *in vivo* incubation resulted in about 50% incorporation of radioiron into the red cells. The cells were washed 3 times with cold saline, then reconstituted to hematocrit of 45%, previously determined to be the mean venous hematocrit of normal mice. The radioactive cell suspension was injected intravenously in volumes of 0.1 ml. The mice were then killed by rapid immersion into liquid nitrogen at intervals of 5 minutes to 6 hours post injection of tracer cells. Tissue samples were obtained(2), weighed, digested in alkali, and assayed for radioactivity with a scintillation counting system. Radioactivity in each tissue was expressed as per cent of injected dose of radioactivity/g of tissues.

Results. Fig. 1 represents a semi-log plot of radioactivity in the circulating blood, following intravenous injection of labeled red cells, expressed as per cent of injected dose/g of red blood cells. The point at each interval represents the mean of 10-12 determinations. Radioactivity/g of red cells exceeds the dose injected because the circultaing red cell volume is less than 1 g, thus when considered on the basis of 1 g, radioactivity associated with red cells is expanded proportionately. The dilution of vascular mixing of injected cells is indicated by an abrupt decline in concentration of blood radioactivity. This decline proceeds in a uniform manner for approximately 15 minutes, at which time blood radioactivity achieves a steady level, essentially maintained through 6-hour period of analysis. The point of transition is taken to represent the time at which vascular mixing of injected cells becomes complete. Fifteen minutes for

vascular mixing of red cells in the mouse agrees quite well with times reported for the dog(3) and human(4). It was expected that because of its accelerated heart rate and shorter circulation time, the mouse would exhibit a more rapid vascular mixing than either dog or human. This apparently prolonged mixing time of 15 minutes may reflect the intravenous injection of a relatively large volume of blood (5% of the blood volume) which would probably require additional time to become fractionated and completely mixed with the endogenous circulating blood.

Distribution of radioactive red cells throughout the tissue circulation is illustrated in Fig. 2. Tissue radioactivity is expressed as per cent of injected dose/g of tissue. The points at each interval represent the means of data derived from 10-12 mice. Since tissues such as lung and spleen are present in small amounts, when expresed on a per gram basis, the value of their radioactivity is expanded proportionately. On the other hand, radioactivity of tissues such as muscle, skin, and intestine which are present in the animal in quantities greater than 1 g, are attenuated when considered on a per gram basis. It will be noted that although concentration of radioactivity in skin, intestine, muscle and lung has reached a steady level within 15 minutes, that in the spleen, kidney and liver has not. To visualize more clearly the characteristics of radioactive accumulation in these tissues, the data presented in Fig. 2 were replotted on a semi-log scale and are shown in Fig. 3. Spleen and kidney exhibit a progressive increase in radioactivity for approximately 1 hour at which time very little further change occurs. During the first hour post injection of radioactive red cells, radioactivity within the liver first rises to a peak and then proceeds to decline. The initial increase in radioactivity of spleen and kidney is considered to represent rate of vascular mixing in these tissues. While these rates may seem slow, it is conceivable that becaue of the sinusoidal nature of the splenic vasculature the distribution of red cells throughout the spleen is somewhat restricted and irregular(5). The variable changes in liver radioactivity following intravenous injection of radioactive cells may also reflect the irregular and periodic flow of blood through the sinusoids of the liver(6). It is possible that a slug of concentrated radioactivity may have become momentarily isolated within inactive sinusoids of the liver thereby imparting a high tissue radioactivity to the organ. Subsequent activation of such sinusoids would permit the concentrated radioactive cells to be flushed out and liver radioactivity would decline. While the kidney does not possess a sinusoidal vasculature it appears to have the ability to restrict distribution of red cells throughout its circulation. This is consistent with the low dynamic hematocrit exhibited by the kidney of the mouse(7).

It is, of course, assumed that all injected radioactivity is securely bound in red cells and remains there so long as the cells maintain their integrity. That concentration of radioactivity in the blood remains essentially constant after the 15-minute post injection period suggests that either this assumption is valid or that any loosely bound radioiron in the injection suspension has been removed from the circulation with 15 minutes post injection.

It appears from our data that 15 minutes post-injection, required for mixing of labeled red cells with the rapidly circulating red cell pool, is also adequate for vascular mixing within skin, intestine, muscle and lung. However, it does not provide sufficient time for complete vascular mixing of red cells within spleen, kidney and liver. In these tissues, approximately 45 minutes to 1 hour is necessary to insure complete mixing of radioactive and non-radioactive red cells.

Summary. Distribution of intravenously injected radioactive red cells throughout tissue circulation of mice was determined. The evidence suggests that distribution of labeled red cells throughout the circulation of spleen, kidney and liver proceeds more slowly than in skin, intestine, muscle and lungs.

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Enhancement of Experimental Atherosclerosis by ACTH in the Dog.* (25420)

Sheldon Rosenfeld, Jessie Marmorston, Harry Sobel and Albert E. White
Inst. for Medical Research, Cedars of Lebanon Hospital, Los Angeles, and Depts. of Medicine and Biochemistry, University of So. Calif.

Prolonged treatment with large doses of ACTH or cortisone is known to elicit a hyperlipemic response in the rabbit(1-3) in man (4), and to a lesser extent in the dog(5). Cholesterol-induced hyperlipemia in rabbits and dogs is heightened markedly during administration of ACTH, cortisone, or hydrocortisone (5-7). The present studies show that prior short-term administration of ACTH enhances development of hyperlipemia in dogs subsequently given a standard thiouracil and cholesterol diet for 6 to 10 months.

Methods. Atherogenic diet: The standard diet consisted of 2 daily doses of 50 mg thiouracil/k of body weight and kibble containing 10% cholesterol (prepared with ether solvent), to provide a daily cholesterol intake of 750 mg/k of body weight(8). To insure complete intake, this diet was fed first, followed by a meat-kibble diet fed *ad lib*. Dogs were divided into 3 groups: *Group A*. Twenty-eight dogs (13 beagles, 3 "Kendall mongrels,"† and 12 ordinary mongrels) were normal controls throughout and fed a meat-kibble diet only. *Group B*. Fourteen dogs (8 beagles and 6 "Kendall mongrels") were maintained on standard atherogenic diet, plus meat-kibble. *Group C*. Seven dogs (4 beagles, one "Kendall mongrel," and 2 mongrels) were given one to 3 injections of 40 units of ACTH-gel subcutaneously at intervals of 6 to 12 days. Two to 4 months later, the 7 dogs were placed on the atherogenic diet. Three additional male beagles received 3 subcutaneous injections of 40 units ACTH-gel at 3 or 4 day intervals. After the third injection, the 3 dogs were started on the atherogenic diet. All dogs but the ordinary mongrels were approximately 1 year of age. All were maintained in kennels with outside runs. Each dog was used as his own control for at least one to 4 months. Each was weighed at 2-week intervals and fed the atherogenic diet individually on a body-weight basis. Blood samples drawn from fasting dogs at 2 to 4 week intervals were analyzed for serum cholesterol(9), phospholipids(10) and alpha and beta lipoproteins (11). At autopsy, tissues were fixed in 10% neutral formalin. Aortas were stained with Sudan IV to demonstrate lipid content of atheromatous lesions(12).

Results. Group A. Without treatment, serum lipid values of beagles and ordinary mongrels were comparable (Table I). Cholesterol and phospholipid values for the "Kendall mongrel" tended to be higher ($p = <0.05$‡) than in the normal beagle. C/P and lipoprotein ratios did not differ significantly between beagles and "Kendall mongrels."

Group B. The atherogenic diet fed 4 to 11 months tended to elevate serum cholesterol, phospholipids, and C/P ratio in all dogs, ($p = <0.05$) and a marked rise occurred in %

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† It has been suggested that breeds of dogs differ in susceptibility to atherosclerosis: beagles are considered to be relatively resistant while a strain, originally mongrels and inbred by Dr. Forrest Kendall, are relatively susceptible.

‡ The rank sum test was used for statistical evaluation of data.

TABLE I. Effect of ACTH Pretreatment on Lipid and Lipoprotein Response to Atherogenic Diet.

Group	Time*	No. dogs ♂	No. dogs ♀	Serum cholesterol and phospholipids: No. determinations†	Chol., mg % ♂	Chol., mg % ♀	P-lip., mg % ♂	P-lip., mg % ♀	C/P ratio ♂	C/P ratio ♀	Lipoproteins: No. determinations†	Lipoproteins: % β ♂	Lipoproteins: % β ♀
Control diet, no ACTH													
A Beagles	1–12	14	14	157	153	163	318	340	.48	.48	94	30	26
Kendall mongrels	1–10	6	4	26	218	217	380	385	.57	.56	16	33	31
Mongrels	2	6	11	22	149	161	295	301	.51	.53	14	34	30
Atherogenic diet, no ACTH													
B Beagles	11	4	4	122	515	510	477	513	1.08	.99	41	48	39
Kendall mongrels	10	3	3	92	721	713	572	605	1.26	1.18	27	46	51
Atherogenic diet, ACTH													
C Beagles	8–10	5	2	61	1322	1325	849	764	1.56	1.73	12	83	67
Kendall mongrels	9	1	0	10	2530		1049		2.41		5	83	
Mongrels	6	2	0	12	988		637		1.55		7	53	

* Time: months on diet indicated, during which determinations were made.

† No. determinations: No. of determinations entering the total male-female averages shown for each column.

As an indication of normal variability in beagles the stand. dev. for cholesterol, phospholipids, the C/P ratio and the % β lipoprotein for males were 33, 53, .07 and 8, and for females 50, 38, .15 and 9, respectively.

beta lipoproteins of beagles and "Kendall mongrels" (Table I, Fig. 1-A and 2-A). The difference between the 2 strains of dog is exemplified in Figs. 1 and 2. This strain difference will be elaborated elsewhere.

Group C. Prior treatment with one to 3 subcutaneous injections of 40 units ACTH-gel§ heightened the hyperlipemic response ($p = 0.001$) of 7 dogs of 3 different breeds maintained for 6 to 10 months on the atherogenic diet (Table I). This effect persisted for 6 to 10 months of the study (Figs. 1 and 2).

Autopsy findings. Dogs in Group C *pretreated with ACTH* and fed the atherogenic diet for 6 to 10 months showed severe atherosclerosis in almost all vessels of the arterial tree. Coronary, thyroid, and cerebral vessels, and the bifurcations and terminal portion of the abdominal aorta were affected most severely. Fig. 3-B demonstrates the high lipid content of atherosclerotic plaques in the lower part of abdominal aorta of a dog from Group C. Death of one dog followed a cerebral hemorrhage near the posterior portion of the circle of Willis. Death of a second animal was asociated with far advanced atherosclerotic lesions in the circle of Willis and all other arteries of the body. Four of the 10 dogs in this group suffered single or multiple cerebral occlusions which produced symptoms similar to those following cerebral occlusions in man.

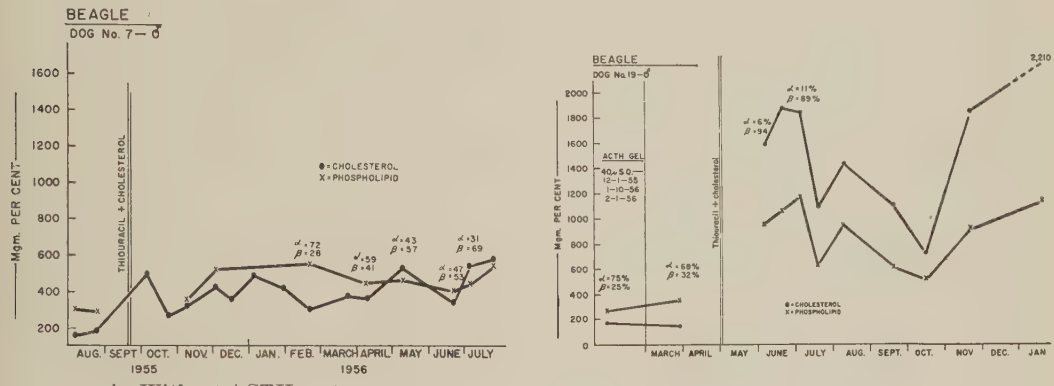
Dogs not *pretreated with ACTH* (Group B) developed scattered, small atherosclerotic plaques, found only in femoral and iliac arteries and in the abdominal aorta and its major branches, as in one case shown in Fig. 3-A.

Discussion. Pretreatment with one to 3 injections of ACTH-gel markedly enhanced subsequent hyperlipemia produced in dogs by feeding a thiouracil-cholesterol diet. This was associated with significant enhancement of the atherosclerotic process in larger arteries.

One of the prerequisites for producing experimental hyperlipmia in the dog has been the establishment of a hypothyroid state. It has been suggested that in the dog the adrenal cortical steroids exert their effect on lipid

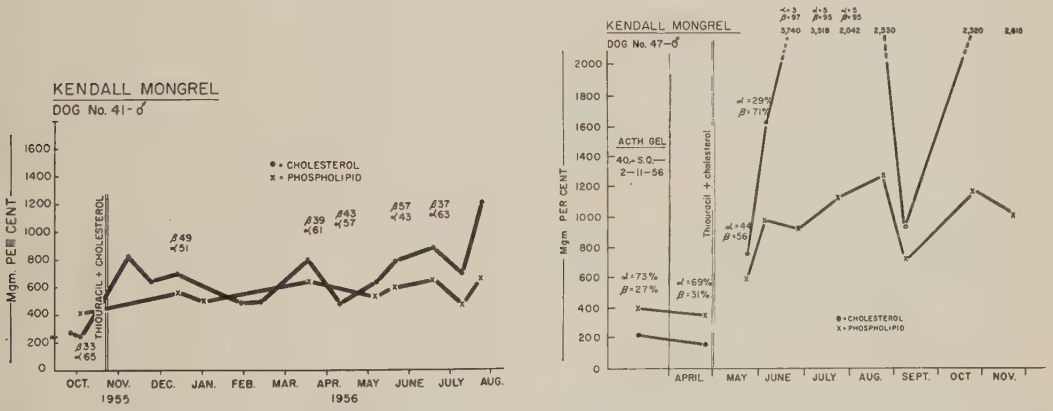
§ ACTH-gel used was made by Organon and Armour Labs.

metabolism, in part at least, through a depression of thyroid function(5). In normal patients, large doses of cortisone have been shown to produce a marked suppression of thyroid function as measured by P.B.I. levels and I^{131} uptake(13). In acute experiments, it has been shown that ACTH and adrenal steroids are capable of reducing rate of release of thyroidal radioiodine in rats and rabbits(14). Furthermore, in the hypophysec-



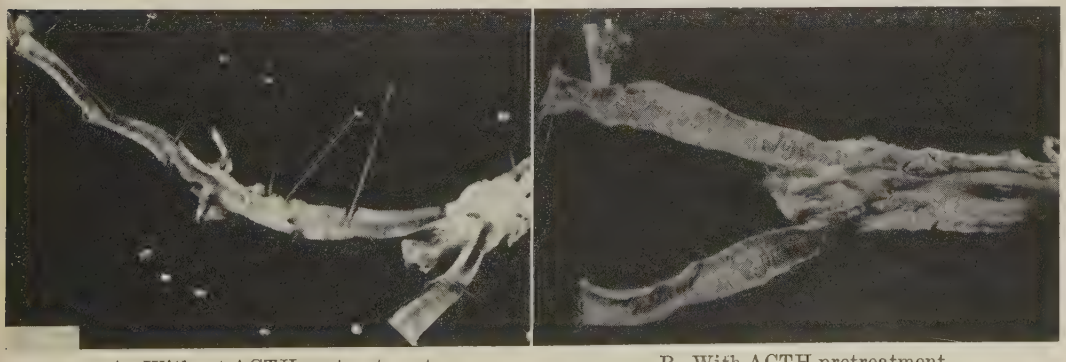
A. Without ACTH pretreatment B. With ACTH pretreatment

FIG. 1. Response of beagle to thiouracil-cholesterol regimen.



A. Without ACTH pretreatment B. With ACTH pretreatment

FIG. 2. Response of "Kendall mongrel" to thiouracil-cholesterol regimen.



A. Without ACTH pretreatment B. With ACTH pretreatment

FIG. 3. Sudan IV-stained aortas obtained from beagles maintained on thiouracil-cholesterol regimen.

tomized rat, ACTH and cortisone have been shown to significantly inhibit the increased thyroidal uptake of I^{131}, known to follow administration of thyrotropic hormone(15).

It has been shown that the level of circulating adrenal cortical steroids is one of the factors which regulates the ACTH content of the pituitary. Adrenalectomy causes a rise in pituitary content of ACTH and hypertrophy of the pituitary(16,17,18). Administration of cortisone produces a depletion of pituitary ACTH. The associated atrophy of the adrenal gland suggests that ACTH secretion is also decreased(18). Furthermore, they showed(18) that administration of ACTH-gel to the rat caused a significant rise in pituitary ACTH, suggesting that lasting derangement of the endogenous ACTH-regulating mechanism may have occurred with failure of high levels of circulating adrenal corticosteroids to suppress further ACTH production. Our preliminary studies reveal a trend for dogs pretreated with ACTH-gel to show increased amounts of urinary 17-hydroxycorticoids. Urinary corticoid excretion for normal beagles averaged 3.7 mg/24 hours (33 determinations); urinary corticoid excretion for thiouracil and cholesterol-fed beagles averaged 3.4 mg/24 hours (40 determinations); and urinary corticoid excretion for ACTH-pretreated dogs averaged 4.7 mg/24 hours (12 determinations).

Summary. One to 3 injections of 40 units of ACTH-gel significantly enhanced the elevated serum cholesterol and phospholipid levels produced by subsequent thiouracil and cholesterol feeding in the dog. This effect was associated with paralytic strokes in 4 of 10 dogs. Autopsy revealed markedly more widespread and more severe atherosclerosis in dogs pretreated with ACTH than in dogs who received only a high thiouracil and cholesterol diet.

The authors are indebted to Drs. Wilfrid Dixon and Frederick Moore for statistical evaluation of the data and Drs. John Mehl and Ross Jacobs for supervision of lipid and lipoprotein determinations.

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Effect of Radiation on Distribution of S^{35}-Labeled Sulfonamide.* (25421)

Mary F. Argus, Judith F. Kane and Francis E. Ray†
Cancer Research Laboratory, University of Florida, Gainesville

Our previous studies on distribution of radioactivity in the animal body, following intravenous injection of fluorene-2,7-di-(sulfonamido-2-naphthalene)-S^{35}, have revealed differences in the localization pattern in tumor-bearing mice, rats and hamsters as compared with tumor-free controls. Concentrations of radioactivity in liver and spleen of control animals are substantially greater than in these same organs of tumor-bearing animals(1,2,3). Autoradiograms indicate that the radioactive material is deposited in cells which have been described by Dunn(4,5) as units of the phagocytic reticulum. Thus, the presence of a tumor in the animal body appears to affect the phagocytic function of the reticuloendothelial system(1). To determine if this is a general stress phenomenon, we followed the distribution of this S^{35}-labeled compound in male and female rats and mice exposed to x-irradiation. Since a total whole-body irradiation of 150 r was shown by Toolan(6) sufficient to inhibit a foreign protein response in Wistar rats, this dosage of radiation was employed.

Materials and methods. 25 (13 male and 12 female) 8-week-old Wistar rats, and 36 (18 male and 18 female) 7-week-old, CAF_1/Jax mice were employed. Six male and 7 female rats, and 12 male and 12 female mice received 150 r whole-body irradiation at the rate of 130 r/min from a 250 KV, 15 ma, General Electric X-ray machine. No filter was added to inherent filter of the tube. Animals were irradiated singly in a vinyl plastic cage. Seven male and 5 female rats, and 6 male and 6 female mice served as nonirradiated controls. Forty-eight hours following irradiation, each rat was administered by tail vein 1.3 ml 0.05 N sodium hydroxide containing 12 mg fluorene-2,7-di-(sulfonamido-2-naphthalene) - S^{35} having a specific activity of 10,000 disintegrations/sec/mg and prepared as previously reported(7). Each mouse received 0.5 ml of the injection solution containing 4.5 mg of the S^{35}-labeled disulfonamide. Six male and 6 female mice were injected 48 hours after irradiation, and 6 male and 6 female mice were injected at 144 hours. Each animal was then placed in individual metabolism cage, and sacrificed 6 hours following administration of the compound. Concentration and per cent recovery of radioactive material in tissues and excreta of animals were determined by methods previously described(8), except for the following modifications. Organs of all rats and total excreta of 15 rats were analyzed individually. For 10 rats, urine and feces were collected separately and individually. For these, feces samples were dried before weighing and 50 ml 1% sodium hydroxide was added for each gram; urine was taken up on filter paper, dried and extracted with 100 ml 1% sodium hydroxide. One ml portions of all excreta samples were plated. Total blood volume was calculated on the basis of 6.7 ml/100 g body weight for rats(9) and 6.32 ml/100 g body weight for mice(10).

Results. Range and average total body weights as well as weights of liver, spleen and thymus at time of sacrifice are given for rats and mice in Table I. That whole-body irradiation of 150 r exerts an effect on young Wistar rats and CAF_1 mice is evident by reduction in weight of spleen and thymus of experimental animals. Reduction in weight of the thymus is statistically significant ($P = 0.05$ or less) for both sexes in both species. For the spleen this decrease is statistically significant in female rats and in male mice 144 hours after irradiation. Similar reductions in weights of spleens of mice at 8 days following 575 r total body irradiation have been reported(11). The present study shows this change even after a shorter time interval and much smaller roentgen dose. On the other

* This investigation supported by U.S.P.H.S. grant and Atomic Energy Comm.

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TABLE I. Weights of Liver, Spleen and Thymus, and Total Body Weights of Irradiated (150 r) and Nonirradiated Wistar Rats and CAF_1/Jax Mice.

	Total body (g)	Liver (mg)	Spleen (mg)	Thymus (mg)
Wistar rats		*Males*		
Range (nonirrad.)	115–145	4496.2–7282.8	411.2–1133.6	375.9–513.5
Avg*	134	5780.8	772.2	437.2
Range (48 hr post irrad.)	100–145	4093.8–6866.4	239.2–1009.2	143.3–265.3
Avg†	126	5593.7	549.0	212.8
Probability‡	$.3 < P < .4$	$\cong .8$	.2	$< .001$
Mean diff. ± stand. dev.	8.0 ± 7.9	187.1 ± 760.2	223.2 ± 163.4	224.4 ± 28.5
CAF_1/Jax mice				
Range (nonirrad.)	19.0–22.0	1154.5–1372.5	89.6–181.8	34.1–45.4
Avg*	20.1	1284.2	133.9	39.3
Range (48 hr post irrad.)	18.5–21.5	1172.9–1401.2	49.5–76.1	16.9–21.5
Avg*	19.8	1294.1	63.7	19.0
Probability‡	$.8 < P < .9$	$> .9$	$.05 < P < .1$	$.001 < P < .01$
Mean diff. ± stand. dev.	.3 ± 1.6	9.2 ± 273.9	70.2 ± 27.7	20.0 ± 3.4
Range (144 hr post irrad.)	19.0–21.5	1202.7–1368.0	50.2–59.2	18.1–27.0
Avg*	20.0	1289.6	54.2	21.5
Probability‡	$> .9$	$> .9$	$.02 < P < .05$	$.01 < P < .02$
Mean diff. ± stand. dev.	.3 ± 1.4	5.4 ± 214.7	79.7 ± 26.8	17.8 ± 4.2
Wistar rats		*Females*		
Range (nonirrad.)	115–166	4155.0–8269.2	440.8–1408.0	284.5–660.0
Avg*	131	6108.0	918.1	486.5
Range (48 hr post irrad.)	105–165	4700.0–8042.2	262.0–404.7	131.0–234.4
Avg†	134	6035.7	330.9	198.8
Probability‡	$.8 < P < .9$	.9	$\cong .01$	$< .001$
Mean diff. ± stand. dev.	3.0 ± 15.6	72.3 ± 775.2	587.2 ± 178.0	287.7 ± 57.3
CAF_1/Jax mice				
Range (nonirrad.)	16.5–20.0	955.6–1158.3	81.7–191.7	31.1–47.7
Avg*	18.7	1061.9	141.5	40.6
Range (48 hr post irrad.)	16.0–20.0	969.6–1186.3	69.6–98.4	17.6–24.6
Avg*	18.5	1102.9	85.3	21.9
Probability‡	$.8 < P < .9$	$.8 < P < .9$	$.1 < P < .2$	$.02 < P < .05$
Mean diff. ± stand. dev.	.2 ± 1.3	41.0 ± 163.1	56.0 ± 30.9	18.7 ± 5.2
Range (144 hr post irrad.)	14.5–17.0	1004.8–1082.3	82.9–90.4	20.8–25.0
Avg*	16.0	1050.8	87.2	22.8
Probability‡	$.8 < P < .9$	$> .9$	$.1 < P < .2$	$\cong .05$
Mean diff. ± stand. dev.	2.7 ± 1.2	9.1 ± 56.6	54.3 ± 32.2	17.8 ± 6.3

* Avg value from 6 animals.
† " " " 7 male and 5 female animals.
‡ Based on null hypothesis for true difference between experimental (irradiated) and control (nonirradiated) groups' values.

hand, total body weight and liver weight are not affected by 150 r irradiation.

Results of complete radioactivity distribution studies 6 hours following intravenous injection of fluorene - 2,7 - di-(sulfonamido-2-naphthalene)-S^{35} are presented in Table II for rats and Table III for mice. Both concentration, expressed as μg compound/g tissue or ml blood or urine, and per cent of administered dose recovered are given. For both species large amounts of the radioactive compound are found in the gastrointestinal tract. The amount eliminated at 6 hours is always less than 3% of administered dose. In those rats where urine and feces were collected separately no difference in rate of elimination is found between experimental and control animals.

In all cases concentration of compound in blood plasma is greater than in blood cells. Differences in concentration of radioactivity in blood between irradiated and control animals might serve as the basis for early detection of radiation damage. The concentration data for blood cells and blood plasma, therefore, were analyzed statistically. No statis-

tically significant difference is found in concentration of radioactivity in blood cells or blood plasma of irradiated rats as compared to non-irradiated rats. This is true for both

TABLE II. Distribution of Radioactivity 6 Hours Following Intravenous Injection of Fluorene-2,7-di-(sulfonamido-2-naphthalene)-S^{35} to Wistar Rats Exposed to 150 r 48 Hours Previously.

Tissue	♂ Control* µg/g	♂ Control* %	♂ Exp.† µg/g	♂ Exp.† %	♀ Control‡ µg/g	♀ Control‡ %	♀ Exp.† µg/g	♀ Exp.† %
Blood cells§	4	.158	7	.181	12	.383	7	.237
" plasma§	9	.432	14	.506	19	.686	15	.543
Liver	126	5.991	163	6.871	251	11.329	221	10.442
Lung	163	1.385	255	1.575	310	2.439	255	1.641
Spleen	43	.279	56	.176	79	.443	81	.210
Kidney	65	.694	97	.948	114	1.268	122	1.147
Thymus	36	.133	59	.093	36	.142	53	.091
Skin	81	18.239	103	21.787	84	18.327	101	22.140
Leg muscle	39	.240	49	.242	45	.274	43	.277
Stomach + contents	18	.316	33	.509	33	1.017	76	1.031
Small intestine + Contents	353	20.407	570	23.937	487	22.059	436	16.190
Large " + "	748	34.773	729	27.913	432	16.822	532	23.803
Carcass	44	23.365	54	25.840	54	26.113	56	29.086
Excreta	239‖	1.231	245‖	1.196	404¶	1.704	183‖	.909
Urine§	60¶		40**		55**		30**	
Feces	10¶		10**		0**		10**	
Total		107.643		111.774		103.006		107.747

* Avg value from 7 rats. † Avg value from 6 rats. ‡ Avg value from 5 rats. § Conc. in µg compound/ml; others expressed in µg compound/g tissue. ‖ Avg value from 4 rats. ¶ Avg value from 3 rats. ** Avg value from 2 rats.

TABLE III. Distribution of Radioactivity 6 Hours Following Intravenous Injection of Fluorene-2,7-di-(sulfonamido-2-naphthalene)-S^{35} to CAF_1/Jax Mice Exposed to 150 r 48 or 144 Hours Previously.

	♂* Control		♂* Exp. 48 hr		♂* Exp. 144 hr		♀* Control		♀* Exp. 48 hr		♀* Exp. 144 hr	
	µg/g	%	µg/g	%	µg/g	%	µg/g	%	µg/g	%	µg/g	%
Blood cells†	100	1.42	49‡	.68	66§	.92	96	1.24	92	1.17	77	.87
Blood plasma†	101	1.42	69	.96	101	1.41	121	1.57	126	1.63	126	1.42
Liver	537	15.22	445	12.78	527	15.07	571	13.23	764	18.38	574	13.44
Lung	1217	3.07	734	1.77	823	2.03	1572	4.27	1475	3.91	1095	2.49
Spleen	357	1.00	258	.37	301	.36	311	.87	389	.71	349	.68
Kidney	420	3.46	336	2.81	506	4.02	418	2.43	429	2.68	389	2.04
Thymus	172	.15	233	.10	227	.11	157	.13	325	.15	275	.14
Skin	372	27.27	389	32.78	346	32.10	378	30.31	406	29.97	430	30.04
Leg muscle	159	.53	98	.28	129	.42	132	.36	169	.59	116	.35
Stomach + contents	202	1.47	299	1.51	265	1.49	238	1.33	213	1.39	183	1.11
Small intestine + contents	705	18.66	413	10.40	394	9.84	366	8.40	407	10.06	343	7.70
Large intestine + contents	337	6.51	1179	21.84	910	16.37	660	9.90	883	15.16	840	13.12
Carcass	181	40.06	166	39.57	174	42.67	162	30.86	197	36.36	184	33.20
Excreta	124	1.38	60	.67	130	1.45	245	2.72	231	2.57	250	2.78
Total		121.62		126.52		128.26		107.62		124.73		109.38

* Each value is avg from 6 animals. † Conc. in µg compound/ml; others expressed in µg compound/g tissue. ‡ $P \cong .001$. § $P = .01$.

TABLE IV. Comparisons of Concentrations of Radioactivity in Liver, Spleen and Thymus of Irradiated (150 r) and Nonirradiated Wistar Rats and CAF_1/Jax Mice 6 Hours Following Intravenous Injection of Fluorene-2,7-di-(sulfonamido-2-naphthalene)-S^{35}.

	Concentration in μg compound/g tissue					
	♂			♀		
	Liver	Spleen	Thymus	Liver	Spleen	Thymus
Wistar rats (48 hr post irrad.)						
Ratio $\left(\frac{\text{avg cont.}^*}{\text{avg exp.}^\dagger}\right)$	.77	.77	.61	1.14	.98	.68
Probability‡	$\cong .4$	$\cong .5$	$.1<P<.2$	$\cong .7$	$>.9$	$\cong .3$
Mean diff. $\pm$ stand. dev.	37 ± 42	13 ± 19	23 ± 16	30 ± 78	2 ± 34	17 ± 15
CAF_1/Jax mice (48 hr post irrad.)						
Ratio $\left(\frac{\text{avg cont.}^*}{\text{avg exp.}^*}\right)$	1.21	1.38	.74	.75	.80	.48
Probability‡	$.1<P<.2$	$.1<P<.2$	$\cong .02$	$.2<P<.3$	$.05<P<.1$	$.05<P<.1$
Mean diff. $\pm$ stand. dev.	92 ± 42	99 ± 57	61 ± 17	193 ± 149	78 ± 105	168 ± 65
CAF_1/Jax mice (144 hr post irrad.)						
Ratio $\left(\frac{\text{avg cont.}^*}{\text{avg exp.}^*}\right)$	1.02	1.19	.76	.99	.89	.57
Probability‡	$.7<P<.8$	$.3<P<.4$	$.05<P<.1$	$>.9$	$.7<P<.8$	$.05<P<.1$
Mean diff. $\pm$ stand. dev.	10 ± 48	56 ± 57	55 ± 22	3 ± 100	38 ± 80	118 ± 43

* Avg value from 6 animals. † Avg value from 7 male and 5 female animals.

‡ Based on null hypothesis for true difference between experimental (irradiated) and control (nonirradiated) groups' values.

males and females. A significant decrease in ability of blood cells to localize the radioactive compound is observed in male mice both 48 and 144 hours following irradiation ($P \cong 0.001$ at 48 hours and $P = 0.01$ at 144 hours). Female mice do not show a significant difference in concentration of radioactivity in blood cells following irradiation with 150 r.

Since previous experiments(1,2,3) comparing tumor-bearing to tumor-free animals revealed a difference in uptake of fluorene-2,7-di-(sulfonamido-2-naphthalene)-S^{35} by liver and spleen, concentrations for these organs from irradiated and nonirradiated animals were compared and analyzed statistically. These data are presented in Table IV. Because irradiation produced a significant decrease in weight of the thymus, concentration ratios and statistics for this organ were also included. Unlike the presence of a tumor, irradiation of 150 r did not affect the uptake of fluorene-2,7-di-(sulfonamido-2-naphthalene)-S^{35} by the liver and spleen. This is shown by the fact that in no case was the P value equal to or less than 0.05. Since autoradiograms(1) have indicated that this S^{35} compound is deposited in those cells that have been described by Dunn(4,5) as units of the phagocytic reticulum, it appears that presence of a tumor in the animal body affects the phagocytic function of the reticuloendothelial system. On the other hand, total body irradiation of 150 r does not produce this effect. This is in keeping with the findings of Barrow *et al.*(12) that in untreated rabbits and in those receiving 500 and 800 r total body irradiation, uptake of colloidal gold by the reticulo-endothelial system was not significantly different when followed up to 30 days.

In one group (male CAF_1 mice 48 hours after irradiation) a statistically significant difference ($P \cong 0.02$) in uptake of radioactivity is noted for the thymus as compared to control animals. The difference represents an increase in ability of the thymus of irradiated mice to localize fluorene-2,7-di-(sulfonamido-2-naphthalene)-S^{35}.

In conclusion, in Wistar rats and CAF_1

mice, whole-body irradiation of 150 r, unlike the presence of a tumor, does not cause a decreased localization of fluorene-2,7-di-(sulfonamido-2-naphthalene)-S^{35} in the liver and spleen. Where a significant difference in the localization of this radioactive compound between irradiated and nonirradiated animals is noted (blood cells and thymus), it is found only for male mice.

Summary. 1. Male and female Wistar rats and CAF_1/Jax mice show a reduction in weight of thymus and spleen 48 hours following whole-body irradiation of 150 r. 2. Unlike presence of a tumor in animal body, this irradiation dose does not affect uptake of fluorene-2,7-di-(sulfonamido-2-naphthalene) - S^{35} by liver and spleen. 3. Significantly different localization of the S^{35}-labeled compound is found in blood cells and thymus of irradiated as compared to nonirradiated male mice.

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Frequency of Spontaneous Fragmentation of Ova In Unbred Gilts. (25422)

PHILIP DZIUK (Introduced by A. V. Nalbandov)
Dept. of Animal Science, University of Illinois, Urbana

Attempts to induce cleavage of unfertilized mammalian ova by physical or chemical means indicated that segmentation or fragmentation can be induced in a high proportion of ova of most species(1). Pincus(2) reviewed the subject of activation of unfertilized ova and Beatty(3) reviewed work on certain spontaneous abnormalities of ova. Evidence has recently been presented indicating a high rate of spontaneous activation in hamster ova (4). The occurrence of fragmented ova in unbred gilts has been noted by Spalding *et al.* (5), Hancock(6) and others but no data have been presented on the relationship between post-ovulatory age of ova and the proportion of ova that were fragmented, nor on frequency and extent of fragmentation.

Materials and methods. This study was made on a group of 31 uniform, unbred gilts killed at various intervals after first or second postpuberal estrus. Estrus was detected by observing behavior of the gilts, and in some cases by observing willingness to "stand" for a teaser boar. Duration of estrus in gilts is 24-48 hours and ovulation normally occurs 30-40 hours after onset of estrus. The ova remain in the oviduct 3 and occasionally 4 days after ovulation. Following slaughter each oviduct and uterine horn was flushed separately with physiological saline solution. The perfusate was examined for the presence of ova with a stereoscopic binocular microscope. Ova were isolated and further examination was made under higher magnification.

Results. Assuming that the number of ovulation points should equal the number of eggs shed, 71% of ova were recovered. An average of 14.7 ovulation points were found/gilt.

Only one ovum of 93 recovered from ovi-

TABLE I. Recovery Site and Frequency of Fragmented Ova in Unbred Gilts.

Estrus to slaughter (days)	No. of gilts	No. of ova			
		Recovered	Oviduct	*In utero*	Fragmented
2	4	41	41	0	1
3	1	9	9	0	0
4	6	62	30	32	23
5	6	79	13	66	46
6	4	45	0	45	39
7	7	65	0	65	57
8	1	12	0	12	12
10	2	10	0	10	10
Total		323	93	230	188

ducts was fragmented. In contrast, 80% of 230 ova recovered from uterine horns were undergoing an orderly degenerative fragmentation (Table I). The proportion of fragmenting ova is positively correlated with post-ovulatory age of ova and it appears that most unfertilized swine ova eventually fragment.

Non-fragmented ova recovered 6 or more days post-estrus were usually cytolyzed. This indicates that at least some ova may degenerate completely without fragmenting.

Fragmented ova usually contained 2-16 fragments and sometimes more (Fig. 1). Ova containing 8-16 fragments of equal size often strikingly resembled normally-cleaving fertilized ova. In many cases ova recovered at 8 and 10 days after estrus resembled shrunken blastocysts.

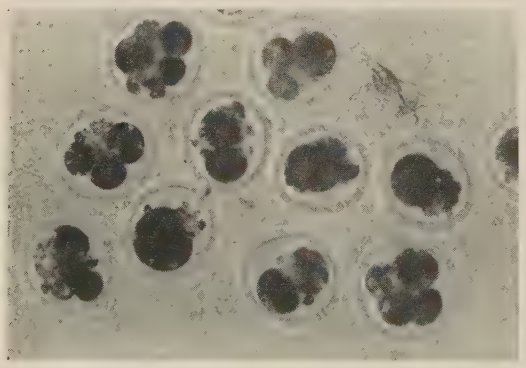

FIG. 1. Fragmented ova recovered from uterus of an unbred gilt 5 days after estrus.

A study of unfertilized pig ova is different from studies on ova of species with comparatively short estrous cycles, in that it is possible to recover unfertilized ova a longer time after ovulation without interference of subsequent estrus and ovulation. This may account for part of the higher rate of spontaneous fragmentation observed in pig ova as compared to ova of most animals with shorter estrous cycles(4). Normal mechanisms for degeneration of unfertilized ova which are inherent in the species may also be responsible for the high fragmentation rate in pig ova.

Because spontaneously fragmented ova, which in some instances resemble normally cleaved ova, are found in a large proportion of gilts and constitute a high proportion of recovered ova, special caution is needed in evaluating ova following *in vitro* manipulations or ova recovered from females following a presumably fertile mating.

Summary. Unfertilized ova were recovered from the reproductive tracts of 31 gilts, 2-10 days after estrus. Examination of the ova revealed that 80% of ova recovered from uterine horns were fragmented. The proportion of fragmented ova increased directly with post-ovulatory age of the ova.

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Intestinal Lymph Flow in Dogs after Endotoxin.* (25423)

HENRY M. BALLIN AND MAURICE W. MEYER†
(Introduced by Maurice B. Visscher)
Dept. of Physiology, Medical School, University of Minnesota, Minneapolis

Because Gram-negative bacterial endotoxin has been shown(1) to produce portal pressure elevation and intestinal mucosal hemorrhage and edema in dogs, it seemed desirable to measure rate of flow of lymph from the intestine in order to throw additional light on the mechanism of endotoxin shock.

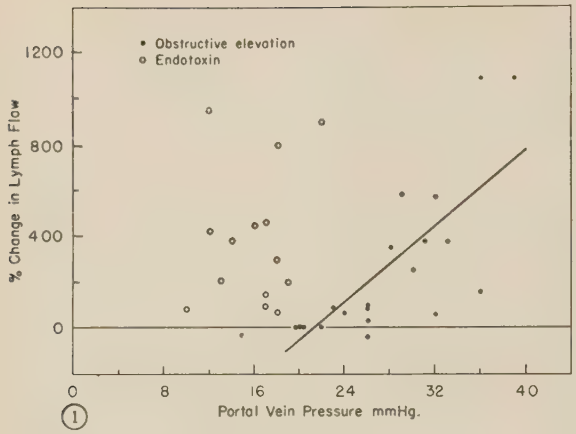
Method. Sixteen adult mongrel dogs of from 9 to 24 kg weight were fasted for a period of 18 to 24 hours, then anesthetized with Nembutal administered intravenously. Endotoxin was injected intravenously either before or after investigation of partial occlusion of the portal vein. Twenty-one experiments in which pressure in the portal vein was elevated by obstruction were performed on 13 dogs. In 11 of these animals, endotoxin was subsequently injected. Systemic arterial pressure (carotid) and portal venous pressure were recorded using the method described by Haddy (2) in which the splenic vein was cannulated following splenectomy. A loop of upper ileum 12 to 46 cm in length was isolated and its lymph vessels were tied off near the central lymph gland, similar to the approach used by Renyi-Vamos(3). About one-half hour later the lymph vessels were distended and could be cannulated according to the method of Lee (4). A single lymph vessel was cannulated and lymph collected from this vessel and its collateral connections. The same cannula (28 gauge needle stud with a short polyethylene tube) was used throughout all experiments in order to keep resistance to outflow constant. Lymph flow was measured as mm^3 per hour from the tip of the cannula. The average drop was measured to be 18.4 mm^3. Mechanical elevation of pressure in the portal vein was accomplished by one of two methods after a portion of the portal vein about 4-5 cm below the liver was freed from all surrounding tissue. Either a screw-clamp fitted with an extension or a long ligature of umbilical tape was placed around the portal vein. The ligature was then passed through a plastic tube, and partial occlusion was achieved by depressing the tube against the vein, similar to the method described by Selkurt and Alexander(5). The clamp or ligature was placed downstream from the tip of the cannula monitoring the portal venous pressure. The results of elevating the portal vein pressure were the same with both methods.

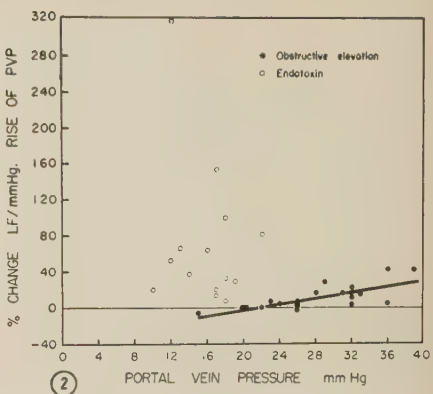
Results. Since pressure in the portal vein usually becomes elevated for about 10 minutes following administration of endotoxin, it seemed desirable to apply equal or greater pressures mechanically for the same period. Time average values were calculated for all parameters measured during this period under both circumstances. Time averages of the pressure data were also computed at specific intervals for 30 minutes following the onset of each experimental condition (Fig. 4). Average control lymph flow rates were 1.53 mm^3/min for the obstructive portal venous pressure study and 1.50 mm^3/min for the endotoxin investigation. Mean lymph flow rates were calculated at various times after conditions were changed. Times of maximal lymph flow and time to first drop of lymph following injection of endotoxin or elevation of venous pressure were noted. Fractions of a drop on the cannula tip at time of change in experimental conditions were removed. The results are presented in Figs. 1 to 4.

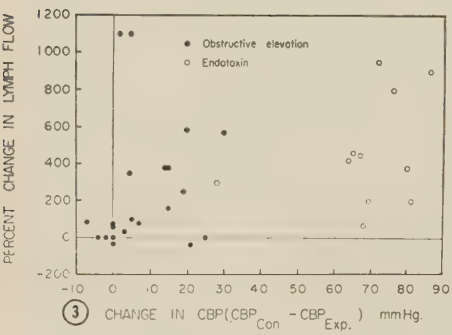
In Fig. 1 the average percentage change in lymph flow was calculated in each experiment and plotted as a function of average portal vein pressure. The method of least squares was used to determine the best fitting line from the observations made during portal vein obstruction. The correlation coefficient in this case was $+0.76$ ($p<0.001$). For the endotoxin effect $r = +0.16$ ($p>0.56$) and the

* The endotoxin used was generously supplied through the courtesy of Dr. W. W. Spink, Dept. of Internal Med., Univ. of Minn. Med. School.

† Postdoctorate Research Fellow of Nat. Inst. of Dental Research.







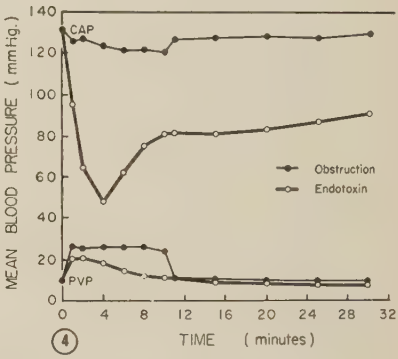


FIG. 1. Relation between percent change in lymph flow and portal vein pressure.

FIG. 2. Percent change in lymph flow (LF)/mm rise of pressure in portal vein (PV) as a function of portal vein pressure (PVP).

FIG. 3. Percent change in lymph flow (LF) *vs* change in carotid blood pressure (CBP).

FIG. 4. Composite graph of pressure tracings under obstructive elevation of portal vein pressure (PVP) and effect of 1 mg/kg (i.v.) *E. coli* endotoxin upon portal vein and carotid artery pressures (CAP).

lymph flow increase was therefore not significantly related to the magnitude of the portal pressure.

In Fig. 2 average percentage change in lymph flow/mm rise of portal venous pressure was calculated in each experiment and plotted as a function of average portal vein pressure. The correlation coefficient in this case was $+0.76$ ($p<0.001$). For the endotoxin effect $r = -0.21$ ($p>0.56$).

When the percentage change in lymph flow is related to the change in arterial pressure (Fig. 3), there appears to be some correlation after endotoxin between fall in arterial pressure and lymph flow rate, but not in the case of portal vein obstruction.

In Fig. 4 average pressure changes are compared in the two studies. Average initial pressure in the portal vein was 9.2 mm Hg, in the occlusion studies and 9.7 mm Hg in the endotoxin experiments. Average portal vein pressure rise during the 10 minutes of mechanical elevation was 16.7 mm Hg while with endotoxin injection it was 6.8 mm Hg. Nevertheless the lymph flow increased 389% after endotoxin injection and only 249% after obstructive elevation on the average in these experiments.

Discussion. Aust and others(6) found a significant sequestration of plasma albumen labeled with I^{131} in the intestine of the dog, 5-7 minutes after administration of *E. coli* endotoxin. According to Heidenhain(7) and Starling(8) ligation of the portal vein, which produces an increase in pressure in the capillaries of the small intestines, resulted in an increase in lymph flow from the intestines. In our findings there appears to be a linear rela-

tion between mean portal vein pressure and the change in lymph flow after partial occlusion of the portal vein (Fig. 1). However, there is not a similar correlation between the increased venous pressure and the change in lymph flow produced after administration of endotoxin. Our data indicate that intestinal lymph flow per unit rise in portal venous pressure increases as the absolute magnitude of the portal pressure by obstructive elevation becomes larger (Fig. 2). This indicates that lymph flow rate is probably not a simple linear function of the ultrafiltration rate, assuming that filtration area and permeability constants do not change. This finding does not necessarily conflict with the conclusion of Pappenheimer and Soto-Rivera(9) that tissue fluid filtration rate is a linear function of pressure above colloid osmotic pressure because lymph flow rate depends upon rate of reabsorption as well as upon rates of ultrafiltration of tissue fluid. Fig. 2 also shows that the correlation between the parameters studied exists for the obstructive elevation experiments but not in the case of endotoxin. Factors other than the elevated portal venous pressure appear to be responsible in major part for the increase in lymph flow after endotoxin.

When the weight of a loop of small intestine was measured in another study(10) under the same experimental conditions, the average percent change in weight/mm rise in portal vein pressure was found to be 0.27 for obstructive elevation and 0.95 after endotoxin. This finding also indicates that in their action upon the intestine there are different mechanisms for the effects of simple obstructive portal pressure elevation and those of endotoxin administration.

It has been suggested that histamine may possibly play a role in the physiological disturbances produced by endotoxin(11). Ráskova and Vanecek felt that they had proved that *Shigella shigae* endotoxin liberated histamine from the tissues(12). It seems likely that some chemical effect of endotoxin, perhaps histamine liberation, is acting on structures other than those causing portal vein pressure elevation, evoking the great increase in rate of lymph flow.

Summary. 1. There is a marked increase in intestinal lymph flow of the dog following injection of *E. coli* endotoxin, and also after partial obstruction of the portal vein. 2. The magnitude of the rise in lymph flow after endotoxin is not significantly correlated with the rise in portal venous pressure. But there is a highly significant correlation between the absolute portal venous pressure level during obstructive elevation and the percentage change in lymph flow. There is a similarly significant correlation between the percentage change in lymph flow per unit rise in portal pressure and the absolute level of portal vein pressure. 3. The increased lymph flow after injection of endotoxin can not be accounted for on the basis of the elevation in portal venous pressure but perhaps is due to the action of substances liberated by some chemical effect of endotoxin which influence lymph flow through some mechanism other than one acting through rise in portal venous pressure.

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Differential and Specific Inhibition of ECHO Viruses by Plant Extracts.* (25424)

Normand R. Goulet,† Kenneth W. Cochran and Gordon C. Brown
Dept. of Epidemiology and Virus Laboratory, School of Public Health, Univ. of Michigan, Ann Arbor

Although recent reviews(1-5) present many examples of inhibition of virus growth in particular complexes of host cell and virus, the chemotherapy of virus diseases remains a special problem. In this connection Bawden(3) observed that "a high degree of specificity, comparable with that possessed by viruses themselves, will be required from a substance that it should prevent the production of one kind of nucleoprotein while not interfering with others sufficiently to damage the synthetic mechanism of the host irreparably." That such specificity can be achieved was demonstrated in the present investigation through the use of plant extracts, some of which were found active against poliovirus in mice(6). Through the use of several ECHO viruses and plant extracts in tissue cultures of monkey kidney cells cytotoxicity of a susceptible virus was inhibited not only without damage to the host cell but also with preservation of the potentiality for responding to several other viruses.

Materials and methods. Monkey kidney cell cultures. Procedures for growth and maintenance of cultures were essentially similar to those reported by Youngner(7,8), modified by use of lactalbumin medium introduced by Melnick(9), and further modified by substitution of calf serum for horse serum. Buffering of medium was accomplished by reinforcement of bicarbonate and phosphate with 0.01M "Tris" (trishydroxymethylaminomethane.) Penicillin (100 units/ml), streptomycin (0.1 mg/ml) and mycostatin (100 units/ml) were incorporated into all cultures. *Viruses.* Thirteen Enteric Cytopathogenic Human Orphan (ECHO) viruses were employed, ECHO–1 through –9, and –11 through –14. These viruses were originally obtained from Dr. J. L. Melnick, Baylor University. Other viruses used included poliovirus type 1, Mahoney strain; Coxsackie B-1, Conn.–5 strain; and vaccinia, Michigan Dept. of Health. Virus was estimated by determining limiting dilution which could initiate infection in 50% of cultures inoculated. The 50% endpoints were calculated by the method of Reed and Muench(10). Virus inocula ranging 32 to 320 tissue culture 50% infectious doses ($TCID_{50}$) were employed in all tests, unless otherwise specified. Hyperimmune anti-ECHO serum was obtained by extensive intravenous and subcutaneous inoculation of young rabbits using high titered monkey kidney tissue culture antigens. *Antiviral extracts.* A number of crude and semi-purified extracts from *Calvatia gigantea,* and other mushrooms and plants, some of which possessed antitumor activity(11,12), were prepared‡ as listed in Table I. Extracts were assayed for toxicity prior to use in virus inhibitory studies. Certain gross cytological changes, such as distortion, pyknosis, swelling or sloughing, were the conditions associated with toxicity to the cultures. Only non-toxic dilutions were employed in the virus inhibitory studies. Except where stated, all cultures were routinely pretreated with extracts for 24 hours at 37°C prior to infection with virus. Antiviral activity was evaluated on the basis of delay, decrease, or prevention of characteristic virus-induced cytopathology when treated cultures were compared to appropriate controls. Effectiveness of these substances was judged by a therapeutic index(13) calculated from the ratio of lowest non-toxic dilution of the substance to maximum viral inhibitory dilution. For example, the therapeutic index of 28.8 for M2 *vs.* ECHO's 4 and 11 is calculated from

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† Present address: Merck Sharp & Dohme, West Point, Pa.

‡ These extracts were prepared and furnished by the late Dr. E. H. Lucas, Dept. of Horticulture, Mich. State University.

TABLE I.

No.	Source and extraction
M1	*Boletus edulis,* fruiting body, chromatographic fraction of ethanol precipitate
2	*Calvatia gigantea,* #644, fruiting body, aqueous extract
3	*Calvatia gigantea,* #644, fruiting body, aqueous pH 6.0
4	*Calvatia gigantea,* #644, fruiting supernatant from ethanol precipitate
5	*Idem,* #643
6	" , #673
7	*Rumex acetosa,* 1:3 aqueous extract of leaves
8	*Hypericum* sp., 1:3 aqueous extract of whole plant
9	*Eleocharis dulcis,* 1:4 aqueous extract of corms
10	*Agaricus placomyces,* 1:2 aqueous extract of ethanol-treated sporophore
11	*Calceolaria crenatiflora,* 1:3 aqueous extract of flowers
12	*Ribes hirtellum,* 1:1 aqueous extract of ethanol-treated fruit
13	*Coprinus micaceus,* ethanol extract of sporophore
14	*Cattleya* sp., ethanol extract of flower
15	*Cattleya* sp., aqueous extract of residue from M14
16	*Calvatia gigantea,* #642, filtrate of blended mycelia and culture broth
18	*Polygonum persicara,* 1:5 aqueous extract of inflorescence, boiled

the ratio of 1/100, the lowest non-toxic dilution, to 1/2880, the maximum viral inhibitory dilution.

Results. Antiviral effectiveness of plant extracts. Results of testing 17 plant extracts against 13 of ECHO viruses are given in Table II. Some extracts showed differential inhibitory activity to a marked degree against some ECHO viruses, and had potential significance. M2, an aqueous extract of the fruiting body of a strain of *Calvatia gigantea,* the giant puffball, displayed significant activity against only ECHO 4, 9, and 11 at concentrations of 1:2880, 1:1200, and 1:2880 respectively. M4, an aqueous extract of the residue remaining from ethanol precipitation of the same species exhibited activity at concentrations of 1:960 and 1:1920 against ECHO 7 and 8 respectively. M14, prepared by ethanol extraction of the flowers of a species of *Cattleya,* exhibited activity at a concentration of 1:480 against ECHO 2. Other extracts were less effective when tested against other ECHO viruses.

Activity of M2 vs. ECHO-4 and 11. In view of the outstanding antiviral effectiveness of M2 against ECHO viruses 4 and 11, it was

TABLE II. Antiviral Activity of Plant Extracts *vs* ECHO Viruses.*

Extract	Lowest non-toxic dilution	Virus inhibited and therapeutic index†
M 1	1:80	E-12, *4*
2*	1:100	E-4, *28.8;* E-7, *4;* E-9, *12;* E-11, *28.8;* E-13, *6*
3*	1:120	E-7, *2;* E-9, *4;* E-13, *4*
4	"	E-1, *4;* E-2, *4;* E-3, *6;* E-4, *4;* E-5, *2;* E-7, *8;* E-8, *16;* E-9, *3;* E-11, *3;* E-12, *2;* E-13, *2*
5	1:10	E-2, *2*
6	1:120	E-3, *3;* E-7, *6;* E-8, *6;* E-12, *6*
7	1:100	None
8	"	E-1, *2;* E-12, *6*
9	1:200	E-6, *3;* E-12, *3*
10	1:100	E-1, *4;* E-11, *6;* E-12, *4*
11	"	E-1, *4;* E-6, *2;* E-9, *3*
12	"	E-9, *3;* E-13, *2*
13	"	E-11, *3;* not tested *vs* E-4, E-8, E-9, E-12, E-13
14	1:10	E-2, *48;* E-8, *4*
15	1:160	None
16	"	E-1, *2;* E-4, *2;* E-6, *2;* E-7, *2;* E-8, *2;* E-12, *2;* E-13, *4*
18	1:200	E-2, *3;* E-9, *6;* E-12, *4;* E-13, *4;* not tested *vs* E-1, E-3, E-5, E-6, E-7, E-8

* ECHO viruses tested 1-9, 11-13; E-14 tested with M2 and M3 only.

† Susceptible virus followed by therapeutic index in italics, therapeutic index was calculated as lowest non-toxic dilution over maximum inhibiting dilution, *i.e.* 1:100/1:2880 = 29.

TABLE III. Inhibition of ECHO Viruses 4 and 11 by M-2 Extract under Varying Conditions of Treatment and of Strength of Virus Inoculum.

Time of M-2 treatment	Virus $TCID_{50}$'s	Therapeutic index ECHO-4	ECHO-11
24 hr before virus	10,000	14.4	
Idem	3,200		9.6
"	1,000	28.8	28.8
"	100	14.4	"
With virus	"	"	
1 hr after virus	"	3.6	

of interest to explore its inhibitory activity more extensively by altering amount of virus and time of treatment. Antiviral activity of M2 varied depending on time of addition and potency of virus inoculum. These results are presented in Table III. Pretreatment of cultures with M-2 for 24 hours completely inhibited ECHO–4 virus up to and including 1000 $TCID_{50}$. Its effectiveness decreased against a challenge of 10,000 $TCID_{50}$ even with 24 hours pretreatment, and when added simultaneously with virus. Further reduction in effect occurred if treatment was withheld for 1 hour after infection of cultures with 100 $TCID_{50}$. Similar results were obtained with M4 against ECHO–11. The possibility that M2 might exert a direct action against free virus was investigated. One aliquot of ECHO 4 was treated with M2 (final concentration, 1:20) while 2 other aliquots each received equal volumes of normal maintenance solution. One of the latter was stored at 4°C and the other 2 aliquots incubated at 37°C for 24 hours. When the 3 samples were titrated for virus content, the titers were 10^{-5}, 10^{-5}, $10^{-4.5}$ for the 4° control, 37° control, and M2 treated samples, respectively. The same procedure was followed for ECHO–11 virus and the titers obtained were 10^{-4}, and $10^{-3.5}$ respectively. No evidence of direct antiviral action was obtained.

Specificity of antiviral activity. Since M2 inhibited selectively the cytopathologic effect of considerable amounts of ECHO viruses 4 and 11, we explored the specificity of this inhibition. Sets of 6 cultures were pretreated with 1/120, 1/240, 1/480, or 1/960 dilutions of M2 for 24 hours at 37°C. The cultures were then divided into 5 groups. Three of the groups were exposed to 100 $TCID_{50}$ of ECHO 4 and 2 groups were exposed to 100 $TCID_{50}$ of ECHO 11. Forty-eight hours later and 24 hours prior to appearance of complete cytopathology in virus control cultures, the fluids were removed, the cultures washed once, and fresh drug added to all treated cultures. At this time large doses of either ECHO–4 (10,000 $TCID_{50}$), ECHO-11 (3,200 $TCID_{50}$) or ECHO–1 (100,000 $TCID_{50}$) were added to one of each group of cultures previously exposed to either ECHO 4 or 11 virus. Data are presented in Table IV. It can be seen from results with the drug control cultures in group 6 that all dilutions of M2 tested protected the cultures against cytopathology from 100 $TCID_{50}$ of either ECHO 4 or 11 viruses for at least 72 hours after initial virus exposure and for the 1/120 dilution for 120 hours. There was also complete protection with stronger concentrations of M2 against re-exposure to those agents even when larger doses of ECHO 4 or 11 were employed (Groups 1, 2, 4, 5). When smaller quantities of M2 (1/480 and 1/960) were employed, cytopathology was prevented on the first exposure to virus, but not when larger doses of virus were added on re-exposure. In all these instances the virus which finally broke through the inhibitory mechanisms was recovered and typed, and was always the second virus. However, addition of ECHO–1 virus to cultures previously protected against type 4, resulted in infection of all the cultures.

An additional experiment was designed to complement above findings. M2 in 1/200 dilution was used to treat cultures which were then exposed to 100 $TCID_{50}$ of ECHO–11 virus, and 48 hours later were re-exposed to 0.1 ml of one of the following: 10^{-1} dilution of ECHO 1 through 7, and 11, as well as poliovirus 1, Coxsackie B1, and vaccinia virus. The challenge doses varied from 100 to 100,000 $TCID_{50}$. In this experiment the fluids were not changed prior to second virus exposure. Cytopathology was prevented when cultures were re-exposed to ECHO 4 or 11, but was complete when cultures were secondarily exposed to a virus not affected by M2. (Table V).

Antiviral activity of M2 in vivo. Demonstration of activity against an ECHO virus *in vivo* is complicated by the lack of readily de-

TABLE IV. Inhibition of Cytopathology* of M-2 Treated Monkey Kidney Cultures after Double Virus Exposures.

Group No.	Cultures pretreated 24 hr with M-2	1st exposure (100 $TCID_{50}$)†	2nd exposure, 8 hr after 1st $TCID_{50}$	Cytopathology Hr after 1st virus exposure 72	96	120	Type of ECHO virus recovered
1	1/120	ECHO-4	ECHO-4	—	—	—	nd‡
	1/240	"	(10,000)	—	+		nd
	1/480	"		—	+		4
	1/960	"		+			4
2	1/120	"	ECHO-11	—	—	—	nd
	1/240	"	(3200)	—	—	—	nd
	1/480	"		—	+		11
	1/960	"		+			11
3	1/120	"	ECHO-1	+			1
	1/240	"	(100,000)	+			1
	1/480	"		+			1
	1/960	"		+			1
4	1/120	ECHO-11	ECHO-4	—	—	—	nd
	1/240	"	(10,000)	—	+		4
	1/480	"		—	+		4
	1/960	"		+			4
5	1/120	"	ECHO-11	—	—	—	nd
	1/240	"	(3200)	—	—	—	nd
	1/480	"		—	+		nd
	1/960	"		+			11
6	1/120	ECHO-4 or -11		—	—	—	nd
Drug control cultures	1/240	"		—	—	+	nd
	1/480	"		—	+		nd
	1/960	"		—	+		nd
Virus controls		"		+			nd

* Designations (—) (+) represent protection and degeneration of cultures.
† Cultures washed once and fresh M-2 added with virus. ‡ Not done.

tectable disease in most laboratory animals (9). However, reports that use of certain antiviral antibiotics altered the immune response to an inoculated virus provided one approach to this problem(14,15). In preliminary experiments, 3 intraperitoneal injections of ECHO virus were sufficient to produce detectable levels of serum antibodies in mice. Accordingly 3 inoculations of 0.5 ml of undiluted, infected tissue culture fluid were given to mice at weekly intervals, one group receiving ECHO–4 virus and the other ECHO–11. Certain mice were treated with 0.01 ml/g of 4-fold diluted M2, which was given intraperitoneally the day before, the day of, and the day after inoculation of antigen. This triad of treatments was repeated with each injection of antigen, while other mice received only M2 and no antigen. At the end of 4 weeks all mice were bled and their sera assayed for antibody content in a monkey kidney tissue culture neutralization test. The results showed that M2 did inhibit formation of antibody since treated mice developed lower titers, 1:6 and 1:12 to ECHO viruses 4 and

TABLE V. Cytopathology of M-2 Treated Monkey Kidney Cultures after Double Virus Exposures.

1st exposure virus,* 100 $TCID_{50}$	2nd exposure virus, 0.1 ml of 10^{-1}	Cytopathology† Hr after 1st virus exposure 72	96	120
ECHO-11	ECHO-1	+		
"	2	+		
"	3	+		
"	4	—	—	—
"	5	+		
"	6	+		
"	7	+		
"	11	—	—	—‡
"	Polio	+		
"	Coxsackie	+		
"	Vaccinia	+		
"	Untreated controls	+		

* 24-hr pretreatment with M-2.
† Cytopathology indicated by — or + representing protection or degeneration.
‡ Evidence of occasional plaques.

11, respectively, than did mice inoculated only with virus, whose respective titers were 1:16 and 1:64. Mice receiving only M2 developed no detectable antibodies.

Discussion. It has been amply demonstrated that viruses respond differently to various chemicals(1-5). In the present investigation a number of ECHO viruses were studied to determine the character of their responses to chemical agents. Differential inhibition with significant individual specificity against certain of these viruses was found in extracts from the mushroom *Calvatia gigantea* (M2 and M4) and from a species of *Cattleya* orchid (M14). In monkey kidney cell cultures M2 inhibited the cytotoxicity of both ECHO 4 and 11 without influencing the cytopathogenicity of several apparently related viruses. Since it was demonstrated that the maximum concentration of M2 employed had no pronounced, direct inactivating effect on these viruses, that possibility may be eliminated in explaining its antiviral activity. No direct evidence was obtained that these preparations specifically inhibit virus multiplication but the available data do indicate that M2 acts cellularly to inhibit the cytopathogenic effect of ECHO viruses 4 and 11 and that it does not block processes essential either to subsequent cytopathogenesis by other selected viruses or for maintenance of host cell vitality. Since both ECHO 4 and 11, individually or consecutively in the same culture, can be inhibited by M2 one can also deduce that they share similar if not identical cytopathogenic processes. These results also indicate that among these presumably closely related enteroviruses, reactions involved in the infectious processes of some are sufficiently distinctive to reveal marked differences in their individual vulnerabilities to chemical agents.

Summary. 1) Comparison of antiviral activity of several plant extracts against several ECHO viruses revealed that M2, prepared from a strain of *Calvatia gigantea* significantly inhibited only ECHO 4 and 11; while M4, a different preparation from the same strain, inhibited only ECHO 7 and 8; and M14, from a species of *Cattleya,* inhibited ECHO 2. The effectiveness of M2 *in vitro* was greatest when the cells were pretreated and when the virus inocula did not exceed 100 $TCID_{50}$. 2) Specificity of the antiviral action of M2 was demonstrated by inhibition of one M2-susceptible virus followed in the same culture by growth of a second M2-nonsusceptible virus. Inhibition continued, however, if the cultures were reinoculated with a second M2-susceptible virus. 3) Activity of M2 was demonstrated *in vivo* by its ability to suppress the antigenicity of an M2-susceptible virus. 4) The results demonstrate strikingly the capacity of the materials to prevent infection of susceptible cells by specific viruses although the cells retain their physiologic competence to support growth of other viruses and the virus is not directly affected by the inhibitory material. Obviously the effect must be directed to modification of reactions essential to viral development but not injurious to functional integrity of the cell.

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Effects of Oxanamide on the Central Nervous System. (25425)

W. L. Kuhn, H. J. Ketteler and E. F. Van Maanen
Scientific Division, The Wm. S. Merrell Co., Cincinnati, O.

Previous investigations of the pharmacological activity of oxanamide* (Quiactin®) characterized it as a central nervous system depressant resembling short-acting barbiturates(1). Our animal studies suggest that oxanamide should be classified with those agents which, pharmacologically, are considered internuncial neuron blocking agents.

Methods. The effect of oxanamide on behavioral changes and reflexes was studied in Swiss mice after oral and intraperitoneal administration. Dose-effect curves were prepared using 60 animals/experiment and the dose which inhibits the righting reflex for more than 10 minutes in 50% of the animals, the Paralytic Dose-50, was estimated. Observations were made of gross behavioral patterns of unanesthetized cats after intravenous administration of oxanamide. The effect of oxanamide on muscle spasticity of decerebrate rigidity was observed in 5 decerebrate cats prepared by the method of Pollock and Davis (2). Muscle action potentials were recorded simultaneously from the triceps brachii. Monosynaptic and multisynaptic reflex arcs were studied in adult cats lightly anesthetized by intravenous administration of 70 mg/kg of chloralose. Polysynaptic reflexes were investigated simultaneously at lumbar cord and bulbar levels of the central nervous system *via* flexor and linguomandibular reflexes. The linguomandibular reflex(3) was elicited by square-wave stimulation of the tongue, with stainless steel needle electrodes using 5 to 6 volt pulses of 0.2 msec. duration at a frequency of 0.5/sec. The flexor reflex was elicited by stimulation of the central end of the cut posterior tibial nerve with pulses of 0.2 msec. duration at a frequency of 0.5/sec. The patellar reflex was elicited by tapping the patellar tendon at a frequency of 1/sec. Movement of the mandible, contractions of anterior tibial muscle and the knee jerk were recorded by spring-loaded muscle levers on a smoked drum kymograph. Compounds were administered intravenously into the exposed jugular vein. Because of prolonged duration of action of oxanamide, the results of only the first injection were used in preparation of dose-effect curves. To remain consistent in our comparison, only the first injection of mephenesin was used in analysis of its effect. Anticonvulsant activity was studied in electrically and chemically induced seizures in male Swiss mice. The maximal electroshock-seizure method described by Toman *et al.*(4) and the electroshock apparatus designed by Woodbury and Davenport(5) were used. Seizures were induced by a current of 50mA for 0.2 second delivered through corneal electrodes. Abolition of the tonic extensor phase of seizures was measured as an anticonvulsant effect. In preliminary experiments the maximal anticonvulsant effect of oxanamide occurred in 15 to 30 min. Dose-effect curves were prepared 20 min after intraperitoneal administration of oxanamide and the ED_{50}'s determined from these. Pentylenetetrazol seizures were induced in mice by rapid administration of 60 mg/kg into the tail vein. An immediate brief clonic seizure was followed by a tonic extensor component in 95 to 100% of the animals. Oxanamide was administered intraperitoneally and pentylenetetrazol was injected 20 minutes later. The ED_{50}'s of oxanamide against the clonic seizure, tonic extensor seizure, and mortality were determined from dose-effect curves. Strychnine seizures were induced in mice by intraperitoneal injection of 2.7 mg/kg of strychnine sulfate. This dose caused tonic seizures and death in 100% of control animals. Dose-effect curves were prepared to determine the ED_{50}'s of oxanamide against tonic seizure and lethal effect of strychnine. Oxanamide was given 20 minutes before strychnine. The effect of oxanamide on conditioned avoidance response in rats was investigated by the method of Cook *et al.*(6). In several of the above investigations similar experiments were

* 2-ethyl-3-propylglycidamide.

conducted with mephenesin for comparison, using the same time intervals. Warm saturated solutions of oxanamide and mephenesin were used for all intravenous injections. Aqueous suspensions in 5% gum acacia were used for intraperitoneal and oral routes. The method of Litchfield and Wilcoxon(7) was used for statistical analysis of results.

Results. Central Nervous System Depression. Oxanamide, administered to mice kept in individual cages, produced a decrease in spontaneous activity in doses above 50 mg/kg intraperitoneally or 100 mg/kg orally. Central nervous system depression gradually increased with higher doses until loss of righting reflex occurred. The PD_{50} (Paralytic Dose 50%) intraperitoneally was 259 mg/kg (19/20 Conf. Limits 242-277) and orally 430 mg/kg (19/20 Conf. Limits 358-516). After a dose of 300 mg/kg intraperitoneally, paralysis occurred within 5 minutes, and average duration of loss of righting reflex was 40 minutes. The pinna reflex was consistently more sensitive to depression by oxanamide than the corneal reflex. The pinna reflex was blocked by doses of 300 to 400 mg/kg intraperitoneally, whereas 500 mg/kg or more were required to block the corneal reflex completely. No signs of hyperactivity or excitability were observed during induction or recovery from the depressant effect.

In experiments in which mice were caged in groups of 5, doses of 200 mg/kg intraperitoneally or 300 mg/kg orally were required to produce significant depression of spontaneous activity.

The acute LD_{50} (Lethal Dose 50%) in mice was 720 mg/kg intraperitoneally and 1,220 mg/kg orally.

Intravenous administration of 60 to 120 mg/kg of oxanamide produced immediate paralysis in 6 cats. This effect disappeared in 3 to 5 minutes. When righting reflex recovered, the animals were alert to external stimuli and would walk when encouraged; however, they remained relaxed and were content even when held in awkward positions. Patellar, pinna, corneal, and photic reflexes remained intact. Injection of 140 mg/kg produced some relaxation of the nictitating membrane in addition to above symptoms. Higher doses were not tested because of insolubility of the compound.

In decerebrate cats, intravenous injection of 20 to 40 mg/kg of oxanamide completely abolished muscle spasticity. Concomitant reduction in amplitude and frequency of muscle action potentials from the triceps brachii was observed. Onset of relaxation occurred immediately after injection. In 3 cases muscle spasticity did not return during 3-hour observation. In one case, muscle rigidity recurred in 30 to 40 minutes after 30 mg/kg.

Inhibition of Polysynaptic Reflexes. Oxanamide attenuated the activity of reflexes evoked in polysynaptic pathways at doses which did not affect significantly monosynaptic reflex arcs. Dose-effect curves were prepared and the PD_{50}'s of the effect of oxanamide on flexor and linguomandibular reflexes were calculated. Similar curves were prepared using mephenesin for comparison (Table I). With doses of 3 to 5 mg/kg, which did not cause obvious depression of reflex activity, oxanamide stabilized those preparations in which activity was erratic or intermittently hyperactive. Inhibition of polysynaptic reflexes occurred with larger doses. The flexor reflex was consistently more sensitive to the effects of oxanamide than the linguomandibular reflex. Doses which produced complete inhibition of these reflexes did not alter the patellar reflex. Both polysynaptic reflexes were equally sensitive to effects of mephenesin. Attenuation of the polysynaptic reflexes by oxanamide or mephenesin could be antagonized by increasing the stimulus strength. A 2-fold increase in voltage usually restored reflex activity to control levels.

Anticonvulsant Activity of oxanamide was compared with mephenesin (Table II). Oxanamide was more potent than mephenesin with respect to antagonism of pentylenetetrazol-induced seizures. This was particularly

TABLE I. Effect of Oxanamide and Mephenesin on Evoked Linguomandibular and Flexor Reflexes in the Cat.

	No. of cats	PD_{50}, mg/kg (19/20 confidence limits)	
		Flexor	Linguomandibular
Oxanamide	12	12 (6.4-22)	23 (12 -41)
Mephenesin	10	12 (6.4-21)	11 (8.1-14)

TABLE II. Antagonism of the Convulsant and Lethal Effects of Electroshock, Pentylenetetrazol, and Strychnine by Oxanamide and Mephenesin in Mice.

	Oxanamide		Mephenesin	
	No. of mice	ED_{50}, mg/kg	No. of mice	ED_{50}, mg/kg
Electroshock				
Tonic seizure	40	125 (113 139)*	40	139 (121-160)*
Pentylenetetrazol				
Clonic seizure	48	145 (126-167)	40	345 (315-378)
Tonic seizure	56	116 (106-127)	56	140 (123-159)
Death	56	102 (90-117)	56	123 (106-143)
Strychnine				
Tonic seizure	40	445 (390-507)	48	>550
Death	56	236 (203-274)	40	320 (274-374)

* 19/20 confidence limits in parentheses.

prominent for the clonic phase of the seizure. Oxanamide was also more potent in antagonizing the convulsant and lethal effects of strychnine. The 2 compounds were equally effective and potent in protecting mice from electroshock-induced seizures.

Conditioned Avoidance Response. In rats, oxanamide had no effect on the conditioned avoidance-escape response (CR) in doses which did not affect motor ability of the animals. In groups of 12 rats/dose level 30, 100, and 300 mg/kg orally had no effect on the CR. After 500 mg/kg the CR was blocked in 4 animals, and the unconditioned response (UR) blocked in one. However, 1,000 mg/kg blocked both the CR and UR in 2 of 12 rats in the group; therefore, this is not considered a specific response.

Conclusion. Although the exact mechanism of action of oxanamide is not known, its spectrum of pharmacological activity indicates that it is similar to mephenesin and related compounds. Central nervous system depression and muscle relaxation without prior hyperexcitability, attenuation of the pinna reflex before the corneal, and antagonism of the convulsant and lethal effects of strychnine are characteristic of mephenesin-like activity. Furthermore, the first 2 effects differentiate the activity of the centrally acting muscle relaxants from that of barbiturates(8,9). Inhibition of polysynaptic neuronal pathways by oxanamide as judged by its effect on the flexor and linguomandibular reflexes also characterizes it as a member of the muscle-relaxant group of drugs.

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Effect of Estrogen on Steroid Levels in Plasma and Urine.*† (25426)

W. L. HERRMANN, I. K. SCHINDL AND P. K. BONDY
Depts. of Obs.-Gyn. and Medicine, Yale School of Medicine

Alteration of adrenal steroid metabolism by administration of estrogenic substances has received considerable attention. The conflicting results, chiefly in animals, have suggested

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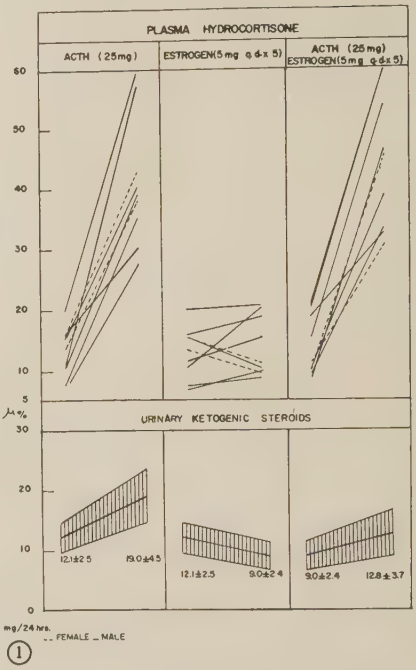
either direct interference with adrenal steroid synthesis, inhibition or stimulation of ACTH (11,12,4). That different parameters of adrenal function, such as adrenal weight, plasma concentration and excretion of Porter-Silber material, 17 ketosteroids, etc., have been studied in various animals and humans and that both synthetic and natural estrogens were used, may have led to these conflicting interpretations. Administration of comparatively large doses of estrogens appears to induce increased levels of plasma corticoids in the human(13,12). However, most authors are hesitant to correlate this with increase in adrenal function. Differences in protein binding of hydrocortisone and in rate of reduction of the molecule have been presented as more attractive explanations(7,13,10). In agreement with this reasoning, it has been shown that total daily production of hydrocortisone by the adrenal is decreased when estrogen is given (9). The present study investigated the effect of approximately physiological levels of naturally occurring estrogenic material on adrenal steroid levels in plasma and urine, and also to determine ACTH responsiveness under such conditions. Through such experiment, more information could be gathered concerning the relation between ovarian and adrenal activity.

Methods. The subjects were 9 apparently healthy individuals, in third decade of life, 6 males and 3 females. Urine was collected during a 48-hour period and stored in deep freezer until processed. Intravenous ACTH (25 mg) was then administered from 9 to 2 p.m. Blood samples were drawn immediately prior to and following ACTH. Another 24-hour urine pool was collected on day of ACTH infusion, starting at 9 a. m. Subjects were then given 5 mg of estrogen by mouth daily (Premarin 2.5 mg B.I.D.) for 5 days. Urine collections were started again on 3rd day of estrogen periods and on 5th day an ACTH test was carried out, as previously described. The entire experiment was therefore divided into 3 parts. Resting level and normal response to ACTH, resting level and response to estrogen, and response to estrogen plus ACTH. The following determinations were carried out on urine: Pregnanetriol (5) ketogenic steroids(8). 17-ketosteroids



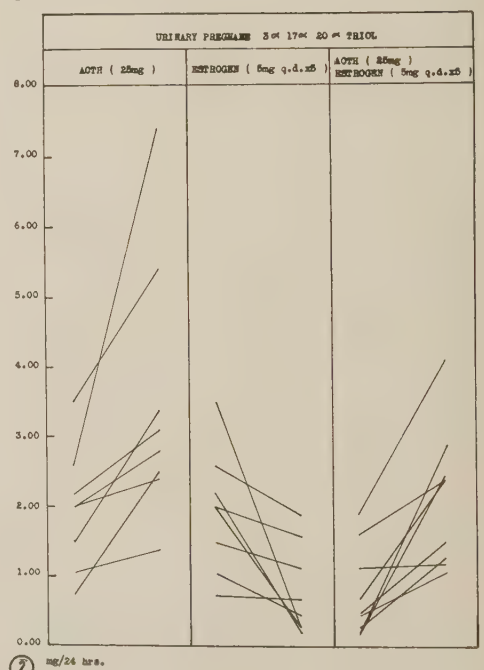


FIG. 1. Response of plasma levels of hydrocortisone (μ/100 ml of plasma) and urinary ketogenic steroids (mg/24 hr $\pm$ S.D. of the mean) to ACTH, estrogen and ACTH plus estrogen.

FIG. 2. Excretion of urinary Pregnanetriol (mg /24 hr) before and after ACTH, estrogen and ACTH plus estrogen.

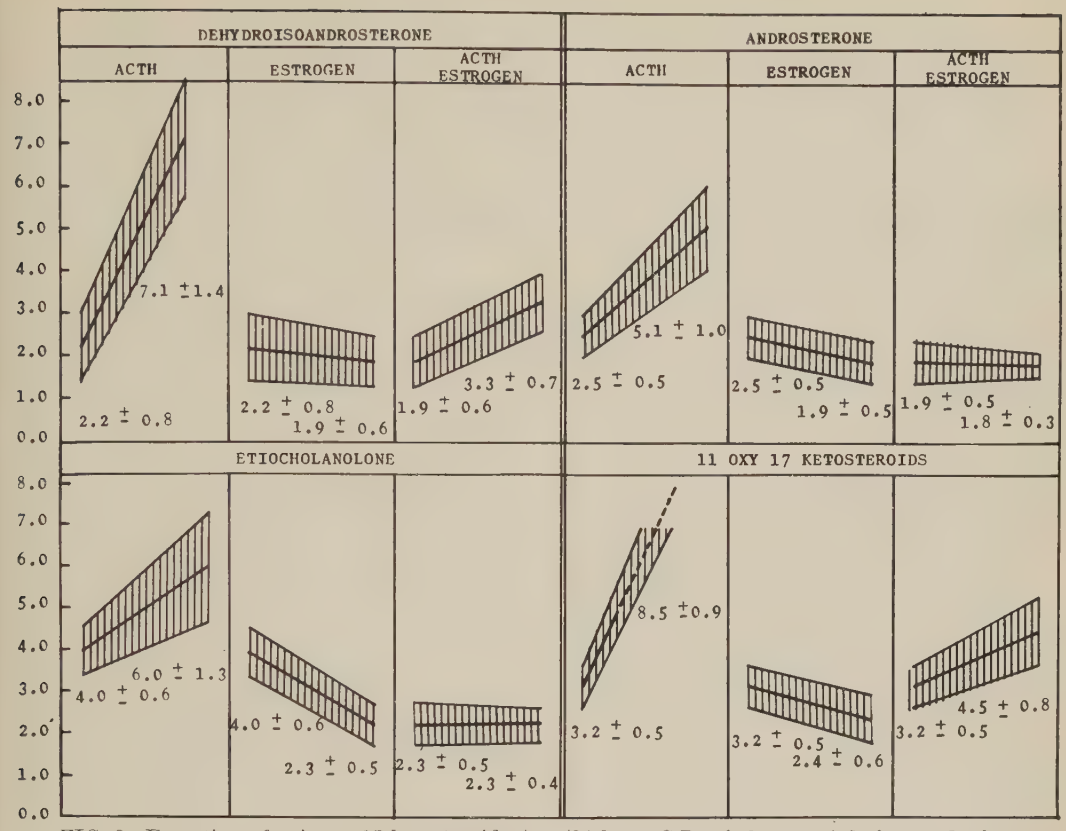


FIG. 3. Excretion of urinary 17 ketosteroids (mg/24 hr ± S.D. of the mean) before and after ACTH, estrogen and ACTH plus estrogen.

were fractionated following β glucuronidase hydrolysis (48 hours at pH 4.5), continuous extraction (at pH 1 and 2 normal for 5 days) and chromatography using alumina column with gradient elution system described by Lakshmanan and Lieberman(6). The binary mixtures appearing in the peaks of the $C_{19}O_2$ compounds were analyzed by the method of Bitman(1). One or 2 peaks in each chromatogram were submitted to infra-red analysis for further identification. Blood samples were tested immediately, by the method of Bondy and Abelson(3) for plasma hydrocortisone.

Results. Hydrocortisone. Plasma levels of hydrocortisone bear some direct relation to adrenal activity. However, under certain physiological and pathological conditions, high values may be found in the absence of proven adrenal hyperactivity. It has also been suggested that patients who have a relative adrenal depression by other standards of adrenal evaluation may still be capable of maintaining a normal plasma level. Results in Fig. 1 must be considered within these limits. There was no significant difference in response to ACTH with or without estrogen. Resting levels did not appear to be different, following estrogen administration.

Urinary Ketogenic Steroids. This method assesses urinary 17 OH-corticoids with the exception of 20-keto-21-methyl steroids. Although it is adequate for clinical purposes, the fact that quantitation is carried out on a crude extract makes it difficult to detect small changes in steroid excretion. This, and the relatively wide spread of normal values, explains the fact that although the observed changes follow the trend of the other urinary steroids, no statistical significance could be attributed to the changes induced in this study. In general, a decreased resting level

was observed following estrogen administration; response to ACTH and estrogen was less than respone to ACTH alone.

Urinary Pregnanetriol. Pregnanetriol is the urinary excretion product of 17-hydroxyprogesterone, precursor of hydrocortisone and perhaps of some C_{19} compounds. Its appearance in urine seems related to adrenal steroid synthesis, as shown by rise of excretion following ACTH and subsequent fall following exogenous cortisone(2). In all subjects studied, excretion of the compound diminished following estrogen administration. Adrenal stimulation with ACTH elicited a response of a magnitude comparable to that seen in untreated patients. The final level found after ACTH and estrogen was lower than after ACTH alone (Fig. 2).

The urinary 17-ketosteroids were dehydroisoandrosterone, androsterone, etiocholanolone and 11 oxy-17-ketosteroids. The latter were estimated from the late peaks in the chromatograms, plus calculated amounts of Delta 9 artifacts running with androsterone and etiocholanolone. As in the case of urinary ketogenic steroids and pregnanetriol, estrogen lowered resting levels of the 17 ketosteroids; and values after ACTH and estrogen were lower than those after ACTH alone (Fig. 3).

Discussion. With the present experiment, estrogen did not significantly affect plasma levels of hydrocortisone, nor their response to ACTH infusions, while it significantly lowered excretion of adrenal steroids. These results are in some respects at variance with results reported earlier. It should be noted that doses of estrogen we used were smaller than those used by other authors. Furthermore, the method used for determination of blood corticoids is more specific for plasma hydrocortisone than those used by various workers.

The changes observed in excretion of 17-ketosteroids and pregnanetriol may be explained as results of direct interference with biosynthesis of these compounds or their precursors by the administered estrogen. However, in the presence of normal plasma levels for hydrocortisone, a retarded catabolism resulting in diminished excretion of all adrenal steroids appears as a more attractive explanation. More specifically, this could be caused by increased protein binding in plasma as shown to occur for hydrocortisone. Further studies on plasma levels of 17 ketosteroids under similar conditions are in progress.

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Rapid Method for Determination of Plasma Fibrinogen.*† (25427)

Dionysios M. Adamis‡ (Introduced by M. Stefanini)
Joseph Stanton Memorial Labs., Saint Elizabeth's Hospital, and Dept. of Medicine, Tufts University School of Medicine, Boston

Since determination of fibrinogen is most useful in hemorrhagic emergencies, the present fairly lengthy methods constitute a definite drawback to its routine use. A technic to determine the level of plasma fibrinogen rapidly and without excessive loss of accuracy would be of obvious value. The procedure described here seems to fulfill these requirements.

Principle of method. Defibrinated plasma does not clot upon addition of thromboplastin and calcium or of thrombin. When, however, a source of fibrinogen is added to defibrinated plasma, clotting time upon addition of thromboplastin and calcium is inversely proportional to concentration of fibrinogen in mixture. *Materials*: (a) *Defibrinated plasma* (substrate reagent). Nine volumes of bovine or of human blood are collected in 1 volume of 0.1 M sodium citrate. Plasma is separated by centrifugation at 2,000 rpm/5 min. Equal volumes of bovine and human plasma are mixed. For defibrination, 5 N.I.H. units of bovine thrombin (Parke & Davis) in 0.1 ml of saline solution are added to 1 ml of plasma mixture. The clotted plasma is left at room temperature for 30 min. and the clot then separated by filtration through gauze. One unit of thrombin in 0.3 ml is again added. The new clot is also removed, the procedure being repeated twice. Completely defibrinated plasma contains optimum concentration of prothrombin and accessory factors. The defibrinated plasma is incubated at room temperature 2 hrs to allow full inactivation of thrombin. When then kept at −20°C in corked test tubes, the substrate reagent is stable and may be used satisfactorily for at least 8 months. It may also be lyophilized and kept at 4°C indefinitely, being reconstituted to original volume of plasma with distilled water at time of use. (b) *Rabbit brain thromboplastin* (Difco) or Permaplastin (C. W. Alban Co.); (c) $CaCl_2$ 0.03 M; (d) *Plasma to be tested*: Nine volumes of blood are collected in 1 volume of 0.1 M sodium oxalate. The plasma is separated by centrifugation at 3,000 rpm/5 min in angle centrifuge. Plasma is absorbed with 50 mg of barium sulfate for 5 min and centrifuged at 3,000 rpm/5 min. Dilutions of the supernatant plasma are prepared with imidazole buffer (pH 7.25). Defibrinated plasma, plasma to be tested diluted 1/5, thromboplastin and $CaCl_2$ all in the volume 0.1 ml are added rapidly in test tube kept in water bath at 37°C and clotting time recorded. Clotting time of mixture is then translated into plasma fibrinogen percentages by means of a curve previously prepared (Fig. 1). Only clotting values should be considered between 20″ and 50″, as the method is most accurate in this range. Occasionally, when fibrinogen concentration is very low it may be necessary to use undiluted plasma or plasma diluted only 1/2.5; with fibrinogen concentration very high it may be necessary to dilute the test plasma to 1/10 or 1/20. In such instances, the percentage values obtained are corrected on the basis of the dilutions used. *Preparation of dilution curve*: Five normal human plasmas were pooled and adsorbed with 50 mg $BaSO_4$/ml as in the test. Supernatant plasma was diluted 1:5 with imidazole buffer and this reagent taken to contain 100% fibrinogen. The reagent was then diluted further to 80, 60, 50, 40, 30 and 20% in imidazole buffer. Values in 5 sets of determinations showed a maximum difference of 1.6 seconds, the higher deviation being noted for the lowest and highest dilutions (Fig. 1).

Reproducibility and standard error of procedure. When dilutions were used giving clotting times between 50″ and 20″, determin-

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‡ Fellow of Mass. Heart Assn.

ations on 3 normal plasmas and several pathological plasmas, using 15 determinations of each, gave a standard error of ± 1.9 sec. When clotting times of undiluted plasmas were longer than 60″, severe fibrinogenopenia was present. Such results are indicated as "no clot." Results have been checked against those with the standard "tyrosine method." Good correlation has been found in patients with normal, elevated or decreased plasma fibrinogen level and in severe hypofibrinogenemia (Table I).

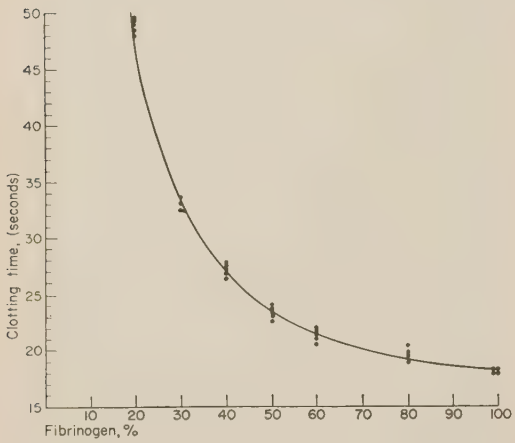

FIG. 1. Correlation of clotting times to plasma fibrinogen percentage values.*

* Clotting times are reported in sec. on ordinate; percentage levels of fibrinogen are reported on abscissa. Thus, clotting time values are readily translated into plasma fibrinogen percentages. The 5 points represent 5 actual experiments using the same system, with plasma pool where fibrinogen content was of 300 mg % by the tyrosine method. Continuous line is avg of the 5 values. As the normal plasma was originally diluted 1:5, the method can be used to study fibrinogen levels from 12 to 1200 mg %. No clot was obtained when plasma was diluted 1:25 or more.

TABLE I. Correlation of Present Method to Tyrosine Method.

No. of cases	Diagnosis	Tyrosine method, mg % (Avg values)	Present method, mg %* (Avg values)
1	Carcinoma of lung	430	388
2	Pulmonary infarction	380	360
2	Arteriosclerosis	400	335
5	Myocardial infarction	292	305
3	Congestive heart failure	272	240
10	Mitral stenosis	368	332
2	Subacute bacterial endocarditis	285	302
2	Rheumatic heart disease	257	267
2	Intestinal obstruction	317	320
1	Fistula in ano	340	464
1	Perianal abscess	365	388
1	Infectious hepatitis	202	235
1	Cirrhosis of liver	100	104
3	Diabetes mellitus	224	220
1	Carcinoma of pancreas	214	168
2	Acute thrombophlebitis	330	360
1	Posthemorrhagic anemia	225	180
2	Fractures	425	447

* Values in mg % are calculated from curve of Fig. 1 where 100% value represented 300 mg % by the tyrosine method. Values were multiplied by 2 or 4 when plasma was diluted 1/10 or 1/20; or divided by 2 or 5 when plasma was diluted 1/2.5 or used undiluted.

Summary. A method is presented for rapid and, under certain conditions, accurate determination of plasma fibrinogen level. The method correlates well with standard tyrosine technic. This method should find its greatest use in rapid determination of plasma fibrinogen level in hemorrhagic catastrophes and especially in hemorrhagic accidents of pregnancy.

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Experimental Penicillin Prophylaxis of Staphylococcal Infection in Rabbits. (25428)

JAMES K. WEAVER* AND GARDNER MIDDLEBROOK

Dept. of Research and Laboratories, National Jewish Hospital, and University of Colorado School of Medicine, Denver

The problem of *Staphylococcus aureus* infections in clean surgical wounds is receiving widespread attention. One method to control this is administration of penicillin. It is currently believed that, in addition to its undesirable side-effects, penicillin is of little or no

* Resident in Orthopedic Surgery, Univ. of Colorado Med. Center.

value for preventing penicillin-resistant staphylococcal infections in clean surgical wounds. To clarify this problem, evaluation of prophylactic penicillin administration in rabbits infected with "penicillin-resistant" staphylococci was undertaken.

Materials and methods. A. *Test organism.* 1. *Characteristics.* A strain of *Staphylococcus aureus* was obtained from the nasopharynx of a ward nurse. It was hemolytic, mannitol fermenting, coagulase positive, and of phage type 80/81. This strain was highly resistant to penicillin by routine disc-drug-susceptibility tests. *In vitro* penicillin resistance also was established by serial tube dilution technics using nutrient broth and reading after 24 hours incubation. With large inocula (5×10^5 to 5×10^7 viable bacterial units/ml) this particular strain was resistant to 250-500 penicillin units/ml. With small inocula (5×10^2 to 5×10^3 viable bacterial units/ml), the strain proved susceptible to 1 penicillin unit/ml, but not susceptible to concentration of 0.5 penicillin unit/ml. This phenomenon of increase in *in vitro* penicillin-susceptibility as size of inoculum decreases is characteristic of penicillinase-producing, resistant staphylococci(1). 2. Staphylococci were prepared for inoculation into wounds in 2 ways: a. *No penicillin* (*np*). Five ml were taken from a heavy 4 to 6 hour culture and grown 2 hours in nutrient broth to which was added 2% glucose. The preparation was aerated during growth in roller tubes turning at 16 rpm. b. The preparation was the same except that 100 units of crystalline penicillin G (Squibb)/ml were added at beginning of 2 hour growth period and additional 100 penicillin (p) units/ml were added after first hour of growth. Both suspensions were then diluted to standard nephelometric density of 20 units (Coleman Nephelo-colorimeter), from this standard, 10^{-4} dilutions in saline were made for inoculation into wounds. 3. *Estimation of inoculum size.* Dose of organisms in each wound was estimated as follows: A sample of suture material identical to that used to close the wound was soaked in the suspension of staphylococci for 30 minutes, then transferred to sterile nutrient broth and allowed to soak for 2 hours. All remnants of the suture used to close the wound were soaked in similar fashion. Aliquots of each broth specimen were plated on nutrient agar and colony counts obtained. Some multiplication occurred during 2 hours of soaking. Difference in number of colonies from the silk prior to closure and the number obtained from suture remnants, after closure, was accepted as a rough approximation of size of wound inoculum. Values for the *np* inoculum were 600, 200 and 50 organisms, and for *p* inoculum 200, 150 and 0 organisms. It seems fair to assume that the dose was *less* than 1000 viable bacterial units in all instances. B. *Technic.* Clean surgical wounds, each approximately 3 cm in length and extending beneath the superficial muscular layer, were done in the backs of albino New Zealand rabbits of similar age and weight (1.5 - 2.0 kg). Infection was consistently produced by a technic similar to that used by Elek(2) in human subjects; namely by soaking silk suture in 10^{-4} dilution of standard suspension (1 to 5×10^4 viable units/ml) and using this suture to close the wound. Fifty cm of 00 non-capillary, braided, Ethicon silk was prepared for each wound closure. The suture was kept completely submerged in 10^{-4} diluted broth for 30 minutes at room temperature, and allowed to drip until free of adherent droplets, and placed immediately in the wound. A 2 layer closure was used with continuous silk in the fascia, and with a running subcuticular stitch to approximate the skin margins. Approximately 12 cm of total 50 cm were used in each wound. Animals were handled in groups of 4 and sites of placement of the differently prepared populations alternated. The type of population placed in each wound was recorded and the records kept in such a way that during postoperative period the observer was unaware of the pattern of wound inoculation. Two animals in each group were treated with procaine penicillin and 2 were not. The treated (*P*) animals received 10,000 units/kg intramuscularly twice daily for 4 days, including day prior to surgery, day of surgery and 2 days after surgery. Wounds were inspected daily. Onset of infection was determined by presence of abscess formation as detected by palpation for induration and fluctuance and by inspec-

tion for erythema. At end of 14 days animals were sacrificed and wounds and viscera inspected. Cultures of all infected wounds were taken at time of autopsy. The infected population was identified in each wound by culture and tests for mannitol fermentation and coagulase production. In selected cases (2 *np* and 2 *p* populations) the strain was further identified by its level of penicillin-susceptibility as determined by tube-dilution technic. To confirm that results obtained were not due to genetic change, the procedure was repeated using a population obtained from a *Pp* abscess in one animal in the initial group.

Results. There were 11 animals in the group which did not receive penicillin (*NP*) (Table I). Each of these animals had 4 wounds placed in its back. One wound was closed with a suture which had been soaked in a suspension of *np* staphylococci, and one wound was closed with suture which had been soaked in suspension of the *p* population. For each of these wounds a contralateral wound was created as a control and closed with sterile silk. There were no instances of infection in control wounds. All 11 wounds infected with *np* staphylococci became infected as did all 11 wounds infected with *p* organisms. These infections became apparent within 6 to 11 days. There was no significant difference in time of onset or clinical appearance of the wounds.

There were 11 animals in the *P* group (which received parenteral penicillin); one animal was eliminated because of wound dehiscence on first postoperative day. There were no infections in control wounds in these animals. In the 10 wounds inoculated with *np* populations there were only 2 infections. These 2 infections were produced in one experiment, and were very early in onset suggesting that the inoculum size may have been larger than in other experiments.

TABLE I. Penicillin Prophylaxis of Staphylococcal Infections.

Animals	Population* (np)	Population (p)
No penicillin prophylaxis (NP)	11/11†	11/11
Penicillin prophylaxis (P)	2/10	10/11

* Staphylococci (np) derived from population not previously exposed to penicillin, and (p) derived from population cultivated with penicillin.
† Wounds infected/wounds inoculated.

TABLE II. Penicillin Prophylaxis of Staphylococcal Infections. Population obtained from a *Pp* abscess, then prepared for inoculation in the same manner as the population in Table I.

Animals	Population* (np)	Population (p)
No penicillin prophylaxis (NP)	7/7†	6/7
Penicillin prophylaxis (P)	2/7	6/7

* Staphylococci (np) derived from population not exposed to penicillin after animal passage, and (p) cultivated in presence of penicillin after animal passage.
† Wounds infected/wounds inoculated.

In animals given penicillin (*P*) wounds which were inoculated with *p* populations became infected in 10 out of 11 instances (Table I). Three infections occurred in the *Pp* sites earlier than infection was apparent in other wounds, including wounds in animals which did not receive penicillin. These *Pp* infections also gave the *clinical impression* of being more erythematous and hemorrhagic. The evidence available did not permit statistical validation of this impression, but was consistent with the findings of Tacking(3).

No evidence of genetic change was detected in bacterial populations recovered from the animals. Those which were tested (in 7 experiments) retained the same level of *in vitro* resistance to penicillin they had manifested prior to animal passage. This was true irrespective of whether they had been passed through a penicillin-treated or a non-penicillin-treated animal. Additional evidence that no genetic change had occurred was obtained in 14 animals that were inoculated with a population obtained from a *Pp* abscess (Table II). The results obtained were similar to those obtained in the original investigation. All groups were combined for final statistical evaluation of the results.

Discussion. Our results indicate that a population of staphylococci which has been exposed to penicillin immediately prior to inoculation has a greater capacity for initiating infection in spite of prophylactic penicillin treatment (16/17) than one of equal "resistance" which has not (4/17), a probability

value$<$0.001. They also indicate that penicillin is effective in preventing wound infections due to "penicillin-resistant" staphylococci not previously exposed to penicillin (18/18 as compared to 4/17) provided the inoculum size is small and the penicillin dosage is adequate ($P<0.001$).

As originally observed by Kirby(1) the resistance of staphylococci to penicillin depends upon ability of penicillinase, synthesized by the bacteria, to inactivate penicillin before the bacterial cell itself is irreversibly injured. The individual cell in such a penicillin-resistant population is relatively susceptible to penicillin. The inoculum must be large enough to provide and maintain sufficient penicillinase in the environment to inactivate a given concentration of penicillin. This is the probable explanation for the difficulty in demonstrating penicillinase production when working with low density populations of staphylococci.

To demonstrate *in vitro,* a difference in penicillin-susceptibility of populations used, the large populations which were cultivated in broth for only 2 hours, with and without penicillin, were studied by tube-dilution technic. No *in vitro* evidence of modification of penicillin-resistance was observed. This seemed surprising in view of the partially substrate-adaptive nature of penicillinase synthesis by staphylococci(4,5). However, it has been observed(6) that synthesis of penicillinase by staphylococci is significantly influenced by the composition of the nutritional environment. Therefore, increased penicillinase production stimulated by previous exposure to the substrate, penicillin, could have occurred in the more complex nutritional environment *in vivo* in spite of the fact that it was not manifested *in vitro.*

In the continuing effort to control hospital infections due to "penicillin-resistant" staphylococci, it appears that control of penicillin in the environment may be as important as the distribution and genetic character of the staphylococci. This appears to be an instance in which the phenotype, as well as the genotype, of a pathogen is important.

Summary. Experimental wound infections with small inocula of staphylococci were regularly produced in rabbits. Prophylactic penicillin was effective in preventing such experimental wound infections due to "penicillin-resistant" staphylococci when size of inoculum was small and when the infecting organisms were derived from environment containing *no* penicillin. Under similar conditions penicillin was not effective in preventing wound infection due to small inocula of "penicillin-resistant" staphylococci which were derived from an environment *containing penicillin.*

The authors acknowledge technical assistance of Charmion Carnes.

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Intimal Lesions in Arteries of Vit. E Deficient Rats.* (25429)

NORMAN M. SULKIN AND DOROTHY F. SULKIN (Introduced by Camillo Artom)
Dept. of Anatomy, Bowman Gray School of Medicine, Wake Forest College

During an investigation on effects of Vit. E deficiency on nerve cells in the rat, it was incidentally observed that in the few instances in which carotid arteries were removed and processed simultaneously with the superior cervical ganglia, the intimal lining of these vessels was characterized by proliferative changes. Since the rat is generally con-

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TABLE I. Lesions in Arteries of Control and Experimental Groups of Rats.

Group No.	No. of rats	Diet	Supplement	No. of animals with arterial lesions	No. of days required to produce lesions
I	76	E-def.		70	280–450
II	20	"	fat	20	150–450
III	"	stock		0	
IV	10	E-def.	vit. E	0	
V	"	"	cholesterol	9	250–450
VI	"	stock	"	0	

sidered to be highly resistant to development of atheromatous arterial lesions, it was considered worth while to investigate this phenomenon in greater detail. Previous attempts to produce atherosclerosis in the rat have met with variable results. According to Okey(1), Page and Brown(2), and Malinow, Hojman and Pellegrino(3), administration of cholesterol does not lead to atherosclerosis. Page and Brown reported that feeding cholic acid and cholesterol to hypothyroid rats elicited severe hypercholesteremia with accompanying fatty liver and lipid infiltration of kidney, heart and aorta, but no evidence of atherosclerotic changes. Malinow, *et al.,* on the other hand, observed "moderately advanced lesions" in arteries of rats which were fed cholesterol together with methylthiouracil. "Moderately advanced lesions" were defined as lesions which showed endothelial or subendothelial proliferation. They also observed similar or more advanced lesions in rats made perinephritic and given methylthiouracil, methylthiouracil and oil, or cholesterol and methylthiouracil. Bragdoon and Mickelsen (4) observed atheromata-like lesions as masses of foam cells. These most frequently occurred on aortic and mitral valves and in endocardium of left ventricle, following intravenous or intraperitoneal injections of lipoproteins obtained from serum of cholesterol fed rabbits. Such changes were never observed in the aorta. These investigators concluded that to date, no one has succeeded in producing atheromatous lesions in aorta of the rat. They explained the lesions described by Hartroft, *et al.* in choline-deficient rats(5) as representing primarily medial degeneration.

Methods. The animals included 126 rats of Wistar (MW-2) strain, weighing 50 g, 150 g and 300 g respectively. Most animals (*Group I,* Table I) were maintained on a tocopherol-deficient test diet which had the following composition: 20% vitamin-free casein GBI, 56% dextrose, 10% lard, 4% salt mixture(6), 10% Brewer's yeast powder together with the following fat-soluble vitamins/100 lb: 523 g cod liver oil, or 68 g carotene in oil, GBI type 3a, and 0.001 g calciferol. Other rats (*Group II*) were placed on above diet to which was added 15 to 20% Vit. E-free fat. This was a hydrogenated cottonseed oil which had been dissolved in saturated ether solution of ferric chloride to oxidize any tocopherol which might be present in the fat. The ether was then removed by a rotating evaporator. *Group III* was placed on stock laboratory diet of Purina Rat Chow, and *Group IV* on tocopherol-deficient diet to which was added pure α-tocopherol. The effects of adding 2% cholesterol to Vit. E-deficient diet (Group V), or to stock diet (Group VI), were also studied. In later studies, 4 rats from 5 different strains (C. F. Nelson, HLA, Long-Evans, Wistar and Sherman) were placed on tocopherol-deficient diet while 2 animals from each strain were maintained on Rat Chow diet. Animals were kept on these diets for various intervals of time from 100 to 480 days before being sacrificed. The tissues were rapidly removed following pneumothorax, fixed in 10% formalin and processed in routine manner. Our study deals with carotid arteries and aorta. These arteries were removed *in toto* and cut into small pieces so that representative sections could be studied from all parts of the arteries. One-half of each piece of artery was set aside for frozen sections and for cholesterol and lipid studies. Blood cholesterol studies were made on 3 control animals and 4 animals that were on the tocopherol-free diet. In these instances blood was drawn directly from the heart following its exposure while the animal was still alive.

Results. The number of rats exhibiting lesions in the aorta and carotid arteries after being subjected to various experimental procedures is shown in Table I. When 15 to 20% tocopherol-free fat was added to tocopherol-deficient diet, arterial lesions occurred much earlier than in animals maintained on the same diet without added fat, the intimal lesions becoming noticeable in a little more than one half the time. Addition of cholesterol to tocopherol-deficient diet, on the other hand, did not alter length of time required to produce the lesions.

In some instances, intimal lesions were quite extensive and completely surrounded the circumference of vessel wall. Such lesions ordinarily extended longitudinally through large segments of the artery and could be observed in most sections of the particular vessel. On the other hand, the majority of lesions were quite localized and appeared in only a few sections of any given segment of artery. Since the lesions in most animals were localized, it was necessary to study large numbers of sections from all of the segments of an artery to determine whether or not arterial alterations existed in an individual animal.

Lesions in the intima varied in size and in nature in different rats as well as in different arterial segments of the same rat. Curiously, there appeared to be no correlation between size of lesions and length of time during which the animal had been on Vit. E-deficient diet.

In several animals, intimal lesions were observed in both carotid arteries and aorta, whereas in others the lesions were limited to the aorta, or to carotid arteries.

Lesions varied from a simple thickening of the endothelium in a localized region comprising only a few sections (Fig. 1) to large, well formed plaques, with fibrotic subendothelial tissues, which protrude into the lumina of the vessels (Fig. 2). In some instances, small lesions extended completely around the circumference of the artery. These could be observed almost throughout the entire length of the respective artery (Fig. 3). The initial thickening of endothelium is due to an increase in height of the normally flattened endothelial cells. This is followed by proliferation of elongated cells resulting in small localized endothelial plaques. In sections in which the endothelium is undergoing active proliferation, this is followed by invasion of connective tissue cells and fibers which arise either

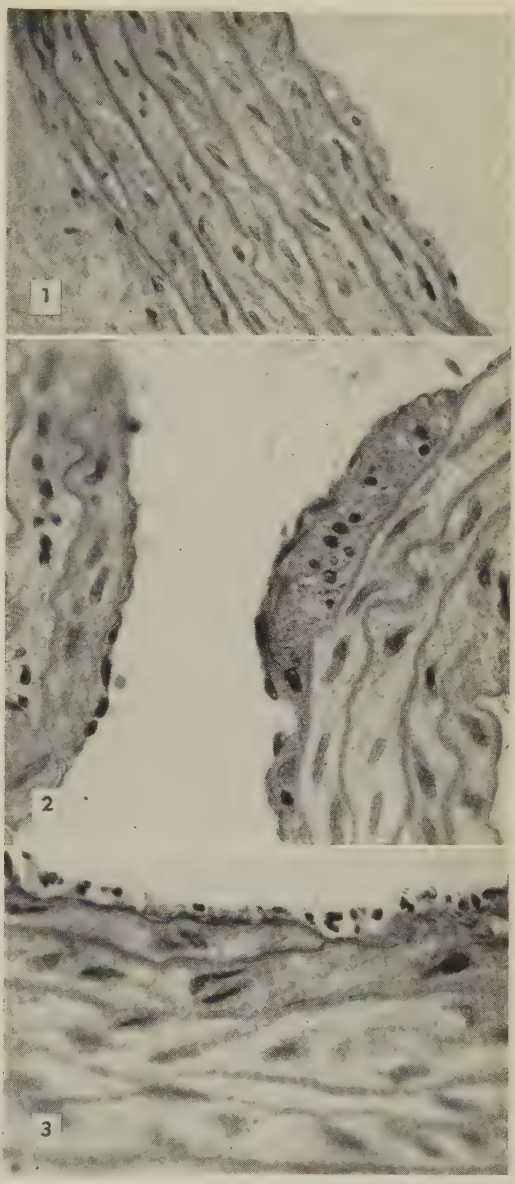

FIG. 1. Photomicrograph of section of aorta from vit. E-deficient rat, showing a small intimal plaque.

FIG. 2. Photomicrograph of section of carotid artery from vit. E-deficient rat showing typical large atherosclerotic plaque.

FIG. 3. Photomicrograph of section of aorta from vit. E-deficient rat showing intimal proliferation which completely surrounds circumference of the vessel.

from subendothelial elements or from the media. This leads to formation of highly organized intimal plaques, some of which are of considerable extent.

Occasionally there is a desquamation of thickened endothelium into the lumen which, in some instances, appears to be still attached to the intima of the artery at one or more points. Moreover, a colorless material is in certain instances deposited below the endothelial cells with subsequent formation of a subendothelium. This is a phenomenon which does not normally occur in arteries of the rat.

Formation of foam cells, which occurs in experimental atherosclerosis in the rabbit coincident with hypercholesteremia, has not been observed in the rat. Moreover, sections of arteries from Vit. E-deficient rats, including sections with intimal lesions, when subjected to Schultz(7) modification of the Liebermann-Burchardt reaction, did not reveal presence of cholesterol. This was true even in animals whose diet had been supplemented with cholesterol. In arteries of rats fed cholesterol in addition to Vit. E-deficient diet, the lesions were identical in both nature and occurrence to lesions of animals fed Vit. E-deficient diet without supplementation. Blood cholesterol levels of 4 rats on high cholesterol Vit. E-deficient diet were essentially the same as those of rats fed the diet without cholesterol.

Study of frozen sections of arteries from rats on various experimental and control diets stained with Sudan black revealed no remarkable differences in sudanophilic lipid material. In most cases, this material was not demonstrable either in the lining of vessels or in the intimal plaques. In only a few instances a small number of sudanophilic granules were present in these areas.

There was no indication that age differences of animals resulted in differences in nature, time of onset, or occurrence of lesions.

Since all observations were made on one particular strain of rats, the possibility presented itself that the results obtained might be due to genetic factors in this strain. For this reason, 4 animals from each of 5 additional strains of rats were subjected to the Vit. E-deficient diet, whereas 2 animals from each strain were placed on the stock diet. No arterial alterations were present in these animals. The group fed the Vit. E-deficient diet exhibited arterial lesions comparable in both nature and extent to those found previously in the larger series of animals, which suggests that the lesions observed were not the result of genetic factors of a particular strain.

Discussion. There is some question concerning the designation of the intimal lesions described here as atheromatous lesions. Although they are characterized by a thickening and proliferation of the intima with eventual formation of intimal plaques there was no marked deposition of lipids, a phenomenon usually considered characteristic of atherosclerosis. For this reason the lesions have been referred to as "intimal arterial lesions."

The effects of Vit. E, or of its deficiency on the vascular system, have been subjected to numerous investigations, the results of which are not always in agreement.

According to some investigators(8,9), increased amounts of tocopherol result in increase in occurrence or severity of atheromatosis and other lesions. Others(10,11,12) state that Vit. E failed to influence atherogenesis or other vascular lesions, while still others(13, 14,15) reported that Vit. E prevents the formation of such lesions or causes their regression. Loewi(16) observed that rabbits and guinea pigs maintained for 7 months on a semi-synthetic Vit. E-deficient diet, develop lesions which resemble atheromata, whereas rats do not.

Although numerous investigators have studied the effect of Vit. E-deficiency in the rat, occurrence of arterial lesions has not been previously observed. There is the possibility that since the majority of lesions are highly localized, their occurrence might not have been noted unless large numbers of sections were studied from any given segment of an artery.

It is of interest that addition of fat (but not cholesterol) to the Vit. E-deficient diet accelerated formation of arterial lesions. Since both saturated and unsaturated lipids were present in fat added, as well as in the diet, these data give no indications concerning the effect of unsaturation on production of the

lesions. Furthermore it should be noted that addition of 15 to 20% fat diluted the diet so that protein content was reduced. This reduction in protein may be related to the acceleration of the lesions.

Lesions differed from those described in cholesterol-fed rabbits(17) or in rats made hypercholesteremic by a variety of procedures (3) or following injections of lipoprotein from serum of cholesterol-fed rabbits(4). In all these instances foam cells, cholesterol and large amounts of sudanophilic lipids were present, with the exception of some described by Malinow, *et al.*(3). The lesions here described were characterized by absence of foam cells and cholesterol, and by presence of only small amounts of lipids.

Summary. 1) The aorta and carotid arteries of rats maintained on a Vit. E-deficient diet for 280 to 450 days were characterized by presence of intimal changes. Addition of 15-20% Vit. E-free hydrogenated fat accelerated formation of lesions. A daily supplement of pure alpha-tocopherol to Vit. E-deficient diet inhibited formation of intimal changes. Addition of cholesterol to the diet had no effect on incidence or severity of the lesions. 2) Intimal alterations varied from a simple thickening of endothelium to large well formed plaques with fibrotic subendothelial tissue.

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Heparin-Latex Test in Rheumatoid and Non-Rheumatoid Serums.* (25430)

G. K. DE FOREST AND J. J. BARBORIAK (Introduced by G. Klatskin)

Dept. of Internal Medicine, and Dept. of Biochemistry, Nutrition Lab., Yale University School of Medicine, New Haven, Conn.

At the first conference on serological reactions of rheumatoid arthritis, in Jan., 1957, sponsored by the Arthritis and Rheumatism Fn., "rheumatoid factor" was defined as "the substance in blood of patients with rheumatoid arthritis and some related diseases responsible for agglutination of sensitized sheep erythrocytes and latex particles in the presence of F II, etc., under appropriate conditions." This definition implies the obligatory presence of an "outside" α-globulin in serological reactions in which the rheumatoid factor participates. It was, therefore, of interest when Gofton *et al.*(1) reported that some polysaccharides, in this case heparin, chondroitin sulphate and hyaluronic acid, may be used in place of α-globulin to sensitize polystyrene latex particles in the latex fixation test. Using a slightly modified procedure, we

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TABLE I. Results of Heparin-Latex Fixation Test.

Clinical diagnosis	No. of patients	No. of positive tests	% positive tests
Definite rheum. arthritis			
Stage 1	20	11	55
" 2	48	36	75
" 3	49	43	88
" 4	4	3	75
Total	121	93	77
Possible rheum. arthritis	24	7	29
Juvenile " "	3	0	0
Diverse collagen diseases	6	1	17
Psoriasis	5	2	40
Spondylitis	8	0	0
Controls	313	20	6

were able to confirm the findings of Gofton's group concerning sensitizing effect of heparin on latex particles used with rheumatoid serums. The present report deals with some results of our work with heparin sensitized latex suspension.

Methods. Reagents and polystyrene latex suspension were prepared and treated according to standard procedure for the F II latex fixation test with the exception that a 0.5% heparin solution, or more precisely a heparin solution containing 500 international units/ml, was used in place of the F II preparation. The latex particles were sent us by the Arthritis and Rheumatism Fn and measured .81 μ in diameter.

Results. The over-all results with the heparin-latex test are given in Table I.

The 121 patients diagnosed as having definite rheumatoid arthritis were subdivided according to stage of disease. The percentage of positive tests increased up to the third stage; the number of patients in the fourth group is not large enough to allow a definite conclusion. The incidence of 77% positive tests in the series with "definite rheumatoid arthritis" compares favorably with similar agglutination tests. In the 24 patients with "possible rheumatoid arthritis," 7 or 29% had positive tests, and none of the 3 patients with juvenile rheumatoid arthritis had serums which reacted. Of the remaining diseases tested, there were no positive tests in a group of 8 spondylitics; 2 out of 5 patients with psoriasis, and 1 out of 6 patients suffering from diverse collagen diseases had positive tests.

Three hundred and thirteen control serums were tested, 20 or 6.4% gave positive tests. Most of these control serums were received from the Serological Laboratory of the Grace-New Haven Community Hospital and came, therefore, from patients with diverse diseases. Over half of the patients whose serums gave false-positive reactions were diagnosed as having some hepatic disorder—mostly cirrhosis or hepatitis. When tested by the *sensitized sheep cell test,* none of the 20 false-positive serums had positive reactions. A review of the electrophoretic patterns of these serums revealed that all 20 had elevated α-globulin levels of over 20%. 7.3% to 22.9% is considered to be the normal range in our electrophoretic studies.

It was then decided to test more high α-globulin serums by both heparin-latex and F II latex fixation tests to find out if a positive reaction is characteristic for heparin-latex only. High α-globulin serums were collected from various sources, all with concentration of α-globulin over 20% of total protein, as determined by paper electrophoresis. Ninety such serums were tested, and the results are summarized in Table II.

The heparin-latex test was positive in a much higher proportion of high α-globulin serums than the F II latex fixation test, 52% as compared with 20%. Interestingly, hepatic disorders made up less than 20% of the group. It is possible, however, that their incidence may have been a little higher, as serums were classified according to a preliminary diagnosis.

Taking the group with hepatic diseases only, 11 out of 16 serums or about 68% re-

TABLE II. Positive Latex Fixation Tests in High γ-Globulin Serums.

Clinical diagnosis	No. of patients	Heparin latex	F II latex
Cirrhosis	11	8	5
Hepatitis	5	3	2
Pneumonia	5	2	1
Colitis	4	1	0
Tuberculosis	3	2	2
Other diseases	62	31	7
Total	90	47	18
% positive		52	20

acted positively with the heparin-latex test, 7 or about 43% were positive with the F II latex fixation test.

It was interesting, however, to note that many of the rheumatoid serums reacting in the heparin-latex test had α-globulin levels well within the normal range.

Little is known about the possible mechanism of the agglutination-promoting activity of heparin. It may have no real biological meaning, but may, as Gofton and co-workers have pointed out, merely represent ability of these strongly negatively charged heparin molecules to react non-specifically with or bring together at a nidus, the patient's own α-globulin and rheumatoid factor. It has been reported that under certain conditions heparin may precipitate fibrinogen(2). In our series, however, serums were used throughout. Certain beta-lipoproteins have also been reported to be precipitated by heparin(3). In the few cases tested in our study no differences in this class of substances could be detected.

Summary. The rather high incidence of false-positive results with serums containing elevated α-globulin levels, limits the practical value of the heparin-latex test for routine testing in rheumatoid arthritis.

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Hexosamine-Collagen Ratio of Skin Biopsies in Patients Receiving Systemic Corticosteroids.* (25431)

E. T. WRIGHT, H. SOBEL AND N. H. NELSON

Vet. Admin. Center, Los Angeles, Calif., Cedars of Lebanon Hospital, and Dept. of Biochemistry and Nutrition, University of So. Calif.

The basic principles which led to the study of hexosamine-collagen ratio (H/C) in human skin biopsies have been described(1). Since collagen is metabolically relatively inert and the hexosamine-containing polysaccharides of the ground substance are part of the metabolic pool, H/C decreases in starvation and after cortisone administration in the rat(2). This effect of cortisone occurs in skin, bone, lung and aorta(3). A technic has been developed for determination of H/C on small dermal punch biopsies from humans and has been applied in a study on the effects of aging(1). It was reported that log H/C falls in a linear fashion with age. This technic has now been applied to patients receiving therapeutic doses of corticosteroids.

Methods. Procedure for obtaining dermal punch biopsies and the analytical methods have been previously described(1). Subjects for the present study were male patients hospitalized on the dermatological service of Veterans Administration Center with a variety of conditions such as psoriasis, exfoliative dermatitis, atopic dermatitis, dysidrosis, etc. No patients were acutely ill nor were they suffering from known metabolic or endocrinological disturbances. The drugs used were prednisone, methyl prednisilone and triamcinalone. These were administered orally in divided doses daily for 2 weeks. The dosage varied depending on clinical requirement of the patient. The average daily dose was 15 to 20 mg for prednisone and 12 to 16 mg for methyl prednisilone and triamcinalone. Biopsies were obtained just prior to and again after 2 weeks of therapy. The area of buttocks from which biopsies were taken was not involved with the patient's skin disease.

Results. A total of 41 sets of biopsies were obtained. H/C values for 40 patients ranged from .042 to .020, initially. One patient, 67-year-old man with atopic dermatitis, had an H/C value of .053, considerably greater than

* This work was supported by grant from U. S. P.H.S.

that expected for this age(1). Following 2 weeks of steroid therapy the following changes were observed: a) 32 exhibited decreased H/C values of 5% or greater. In 29 of these the losses did not exceed 30%, in 3, decreases of –43%, –63% and –69% were obtained (the latter due to the individual mentioned above); b) 6 had changes of less than 5%;† c) 3 had increased values (+23%, +10%, +34%). It is of additional interest in the case of the latter that pretreatment H/C values were .027, .020, .023 respectively, values expected for aged individuals(1). The latter 2 H/C values were the lowest pretreatment values in the series. The significance of the increased values following corticoid treatment is not clear and has not been observed in rats(2,3). The mean percent change in H/C values for 35 subjects (exclusive of the 3 who exhibited exceptionally large decreases and the 3 who exhibited increases) was –12.9% ±1.5%.‡

Discussion. In general H/C of dermal punch biopsies decreased following corticoid therapy. This had been previously observed in the rat following starvation and administration of cortisone(2). Since the dose of corticoids administered to patients was based upon clinical requirements, the effect of graded doses upon H/C could not be determined. The wide range in response of H/C seemed to be due to individual variation and not to choice of steroid employed. There appeared to be no correlation between clinical response of patient to the corticoid and decrease in H/C. Since the decrease in H/C in the rat following cortisone administration precedes a large decrease in body nitrogen, the change in ratio in the human might likewise presage other metabolic events. This suggests employment of this technic as an adjunct in tests used for the screening of new steroid therapeutic agents prior to clinical application. Decrease in H/C occurs in the rat in other connective tissue-rich organs in addition to the skin(3). The findings suggest that in man the composition of the ground substance of lung, blood vessels and bone is affected as well.

Summary. Dermal punch biopsies were obtained from 41 subjects prior to and following 2 weeks of daily medication with prednisone, methyl prednisilone or triamcinalone. The hexosamine-collagen ratio (H/C) was determined. In 32 subjects decrease in H/C values of 5% or greater were observed. The mean change in H/C in 35 patients following corticoid treatment was –12.9% ±1.5%.

Analysis of skin punches for hexosamine and collagen was carried out by George Bonorris.

† This classification was established because in previous experience obtained with multiple biopsies from rats and dogs taken concurrently or on different days from the same region of skin, values of H/C did not exceed ±5% of the mean. It was considered clinically unjustifiable to obtain more than one punch at a time in human subjects and consequently this same technical error is assumed.

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Local Action of Oxytocin on Mammary Glands of Postpartum Rats After Litter Removal.*† (25432)

JOSEPH MEITES, C. S. NICOLL AND P. K. TALWALKER
(With technical assistance of C. A. Goerner)
Dept. of Physiology and Pharmacology, Michigan State University, East Lansing

Petersen(1) first suggested that oxytocin may stimulate release of prolactin by the anterior hypophysis and thereby initiate lacta-

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tion. This was challenged by Meites and Turner(2,3) who reported that oxytocin does not induce prolactin discharge or initiate lactation in laboratory animals. Both oxytocin and prolactin are released by the suckling stimulus in lactating rats (see 4), as indicated by ejection of milk and decrease in pituitary prolactin content. There is no evidence, however, that release of prolactin following suckling is dependent on stimulation by oxytocin (4,5). In contrast to its inability to initiate lactation, oxytocin has recently been shown by Benson and Folley(6) and Benson *et al.* (7) to maintain mammary secretion in postpartum rats after litter removal. This was confirmed in our laboratory(8,9) and by McCann *et al.*(10). Inasmuch as prolactin also maintains mammary secretion in such rats (see 4), Benson and Folley(6) and McCann *et al.*(10) reaffirmed that the effects of oxytocin were mediated through inducing prolactin release. Absence of any direct evidence for the latter viewpoint has led us to examine the possibility that the action of oxytocin in maintaining mammary secretion in postpartum rats after litter removal is due to its well-established ability to induce ejection of milk from the alveoli into the larger ducts.

Methods. Five groups of 5 rats each (Carworth strain), weighing 200-250 g, were bred and on 4th day after parturition their litters were removed. Mammary secretion was subsequently maintained by injecting them subcutaneously as follows: Group 1, 1 mg prolactin‡ daily for 5 days; Group 2, 2 mg prolactin daily for 5 days; Group 3, 2 mg prolactin daily for 9 days; Groups 4 and 5, 1 IU oxytocin§ twice daily for 5 days. About 16 hours after last injection, a biopsy was taken from the right inguinal mammary gland of each rat under light ether anesthesia, and 30 minutes later Groups 1-4 received an intraperitoneal injection of 2 IU oxytocin and Group 5 an intraperitoneal injection of .85% saline. Ten minutes later, all rats except Group 2 were killed and remainder of right inguinal mammary gland was removed. From Group 2, biopsies were taken from right inguinal mammary gland at 10 minutes and 1, 2, 4 and 8 hours after oxytocin injection. Mammary tissues were sectioned and stained by standard histological methods, and examined microscopically.

Results. More mammary secretion was seen in Groups 1 and 2 after 5 days injection with prolactin than in Group 3 after 9 days of similar treatment. About the same amount of milk was seen in mammary alveoli of rats injected with oxytocin for 5 days (Groups 4 and 5) as in Groups 1 and 2, but the mammary ducts were better filled with secretion in the former rats. Epithelial cells lining most of the alveoli of prolactin and oxytocin-injected rats were flattened by the secretion, and blood capillaries surrounding alveoli appeared closed.

Gross and histological appearance of the mammary glands 10 minutes after intraperitoneal injection of oxytocin was profoundly different from biopsies taken previously. When fresh mammary glands were first removed and viewed macroscopically through transmitted light, the ducts leading to the nipples were widely distended with milky secretion, in contrast to the very thin ducts in mammary glands prior to oxytocin injection. Microscopically, the mammary alveoli of oxytocin-injected rats were largely empty of secretion and were shrunken or collapsed, while the ducts were heavily swollen with secretion. In many sections, the distended ducts appeared to take up as much or more space than the alveoli in the mammary parenchyma. Some alveoli had ruptured and the secretion had apparently escaped into the stromal tissue spaces. In Group 5, injected with saline, the alveoli remained filled with secretion and could not be distinguished from control mammary biopsies taken prior to saline administration. Representative mammary sections are shown in Fig. 1-4.

In Group 2, somewhat greater emptying of alveoli appeared to occur after one hour than 10 minutes after intraperitoneal injection of oxytocin. Subsequently, little change was observed until the 8th hour, when a moderate amount of secretion had reappeared in the alveoli and the ducts were less distended. Ap-

‡ Prolactin (1 mg = 20 IU) was kindly donated by Endocrinology Study Section, NIH.

§ Oxytocin was supplied through courtesy of Dr. D. A. McGinty, Parke, Davis and Co.

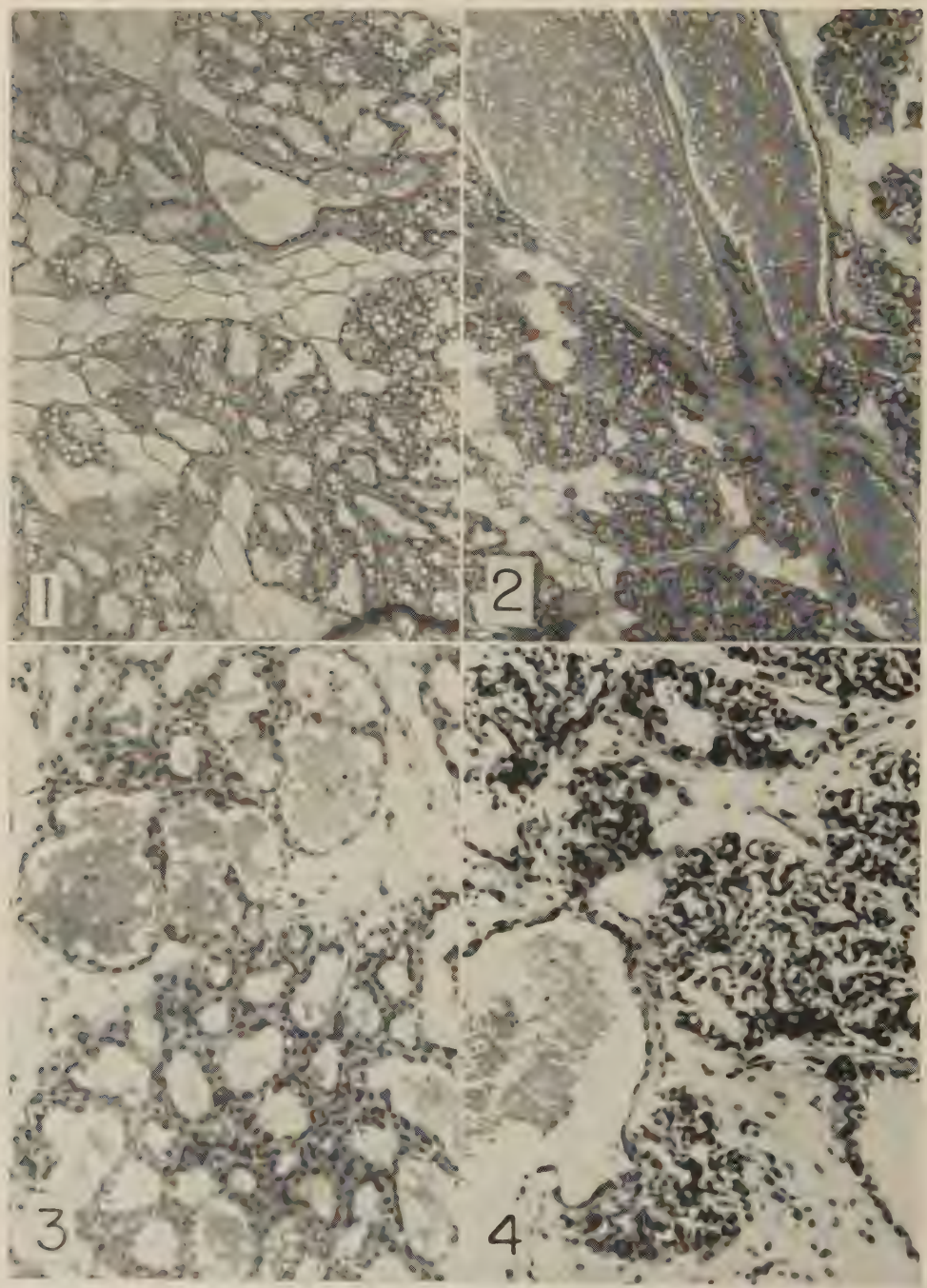

FIG. 1-4. Histological sections of mammary glands from postpartum rats after litter removal: (1) From rat given 2 mg prolactin daily for 5 days. × 45. (2) From same mammary gland 10 min. after intraper. inj. of 2 IU oxytocin. Note shrunken alveoli with relatively little secretion in contrast to engorgement of ducts. × 45. (3) From rat given 1 IU oxytocin twice daily for 5 days. Note distended alveoli with flattened epithelial cells. × 135. (4) From same mammary gland 10 min. after intraper. inj. of 2 IU oxytocin. Note collapsed alveoli and milk in ducts. × 135.

parently, more than 8 hours was required to refill the alveoli after oxytocin administration. Linzell(11) observed that alveolar diameter in mammary glands of mice increased 10-fold 6 hours after milk removal by suckling litters. No comparable increase in alveolar diameter was apparent in the mammary gland of unsuckled rats after oxytocin administration.

Discussion. These results show that in postpartum rats after litter removal, oxytocin induces ejection of milk into the larger duct, just as it does in rats from which milk is normally removed by litters. It is probable that secretion from ruptured alveoli is ejected into stromal tissue spaces, although no such material was detected microscopically and may have been lost during staining procedures. Selye(12) did observe milk in the stroma of suckled rats from which milk could not be removed because the main ducts had been cut. Emptying of alveoli is believed to reduce the pressure against epithelial cells and the blood capillaries surrounding them, permitting more synthesis of milk under stimulus of circulating prolactin and other hormones.

The fate of ejected secretion in ducts is not clear. It is probable that some is reabsorbed into the circulation, and some may flow back into the alveoli. Linzell(11) observed the latter phenomenon following oxytocin injections in anesthetized mice from which milk was prevented from leaving the nipples. Rate of milk secretion by mammary glands of postpartum rats treated with prolactin or oxytocin after litter removal, is obviously much less than if milk is removed. Continuation of mammary secretion in non-suckled rats is believed to depend on establishing an equilibrium between rate of milk produced and that reabsorbed into the circulation.

Benson and Folley(6) do not believe that the action of oxytocin in maintaining mammary secretion in postpartum rats after litter removal is a direct one on the mammary gland, since they were unable to observe milk secretion following oxytocin injections into hypophysectomized rats. However, milk secretion cannot be maintained without stimulation from prolactin and ACTH(4). Recently, we reported that when oxytocin and prolactin were injected together into postpartum rats after litter removal, mammary secretion was maintained at a higher level and for a longer period of time than when prolactin or oxytocin were given alone(9,13). If oxytocin acted through increasing prolactin secretion, then oxytocin injections should have been ineffective when prolactin was also administered, and vice versa. Cortisol was found further to supplement the actions of these 2 hormones and permitted lactation to continue for 75 days. This suggests that the action of oxytocin in maintaining mammary secretion in postpartum rats after litter removal is a direct one on the mammary gland, and is not mediated through prolactin or other hormones.

Summary. 1. Since no convincing evidence exists that the action of oxytocin in maintaining mammary secretion in postpartum rats after litter removal is due to prolactin release, attempts were made to determine whether its effects were exerted directly on the mammary gland. After maintaining milk secretion in 25 such rats for 5 or 9 days by daily injections of prolactin or twice-daily injections of oxytocin, a mammary biopsy was taken from each rat. They were then given an intraperitoneal injection of 2 IU oxytocin or physiological saline, and 10 minutes later all except 5 rats were killed and the remainder of the previously biopsied mammary gland was removed. Mammary biopsies from the remaining 5 rats were taken after 10 minutes and also 1, 2, 4 and 8 hours later. 2. Histological examination revealed that prior to intraperitoneal oxytocin administration, most mammary alveoli contained considerable secretion and the ducts were relatively thin, whereas 10 minutes after oxytocin the alveoli appeared shrunken or collapsed with little or no secretion, and the ducts were widely distended with milk. No notable increase in alveolar filling followed until the 8th hour after oxytocin injection. Saline had no effect on mammary alveoli and they remained filled with secretion. 3. Ejection of milk from the alveoli into larger ducts by oxytocin is believed to remove the pressure on epithelial cells which secrete milk and on blood capillaries surrounding them, permitting synthesis

of more milk by circulating prolactin and other hormones. Milk ejected into ducts is believed to be gradually reabsorbed into the circulation, and some may flow back into the alveoli. These local effects of oxytocin on the mammary gland are believed to account for its ability to maintain mammary secretion in postpartum rats after litter removal.

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A Modified Bile Return System in the Dog.* (25433)

BENNETT J. COHEN (Introduced by Ralph R. Sonnenschein)
Dept. of Physiology, School of Medicine, University of California, Los Angeles

Steatorrhea in bile fistula dogs was reduced substantially following hourly return of the dogs' own bile to the duodenum(1). The steatorrhea was greater when bile was returned every 4 to 8 hours, even though total cholic acid returned was the same. Thus, it appears that bile must be in the intestine nearly continuously to effect normal fat absorption. However, complete correction of the steatorrhea of bile deprivation by continuous return of bile has not been demonstrated. A modified Rous-McMaster bile fistula preparation(2) is described in this report, permitting free entry of bile to the duodenum, and restoration of normal fecal fat levels following bile deprivation.

Methods. Duodenal and gall bladder cannulas were prepared for implantation with Tygon tubing (1/16″ wall, 1/4″ and 5/16″ O.D. respectively, overall length approximately 12″ each). Two Tygon collars (Fig. 1; a,b) and an abdominal wall brace (c,d) were cemented on each cannula with Tygon paint. The cannulas were then coated with a silicone preparation (Siliclad, Clay Adams, Inc.) to minimize bile sedimentation on the inner wall. Sterilization was accomplished by soaking in benzalkonium chloride (Zephiran, Winthrop Stearns, Inc.). Clogging of the duodenal cannula tip with intestinal contents was obviated by placing 1½″ slits bilaterally in the intraduodenal segment (e). The surgical procedure was carried out aseptically on mongrel female dogs weighing 9 to 18 kg with due regard for the requirements of proper animal care. The common duct was ligated and sectioned. The cannulas were anchored with a double row of purse string sutures and brought through stab incisions in the right abdominal wall (Fig. 1). The bile cannula extended into the base of the gall bladder about ½″ (f), in a line with the cystic duct. A male luer fitting (g) and a stethoscope fitting (h) were inserted. During bile collection periods the duodenal cannula was capped and bile was drained into a rubber balloon. During bile return periods the 2 cannulas were connected to reestablish the enterohepatic biliary circuit. A many-tailed binder covered the cannulas. A balloon bag was pinned to the binder during collection periods. 30-50 ml of isotonic saline was in-

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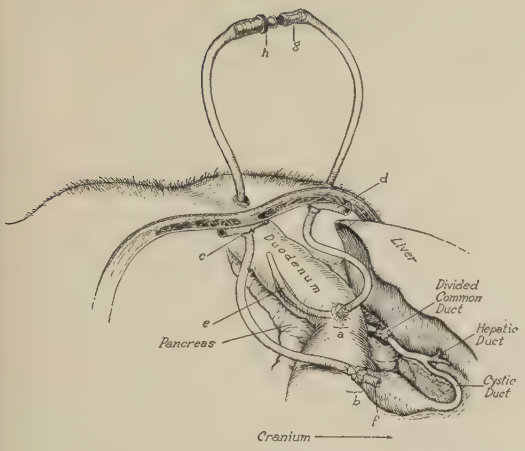

FIG. 1. Schematic representation of cannulas implanted in duodenum and gall bladder.

jected through the duodenal cannula every morning and evening to assure its patency. Test meals, fed once daily, starting approximately one week after operation, were completely consumed. They consisted of 300 g of canned dog food (Friskies, Carnation Co.) and 2 g of brewers yeast, to which varying amounts of oleic acid or triolein were added (Table I). Feces were collected and refrigerated for the last 3-5 days of 5-7 day test periods. Fecal fat excretion was then estimated on duplicate aliquots of the total blended fecal sample as the petroleum ether soluble material extracted in 7 hours(3).

Results. The experiments summarized in Table I were carried out on 2 groups of animals: on dogs which it is assumed suffered no bile deprivation since the gall bladder and duodenal cannulas were joined at time of operation and remained connected during the subsequent month of tests; and, on dogs following one to three 5-7 day bile deprivation periods. None of these animals became visibly jaundiced during these tests.

Inasmuch as large doses of oleic acid are poorly tolerated even by normal dogs(4), the slight but significant increase in level of fecal fat when 25 g of oleic acid was fed is not surprising. However, on all other regimes no significant difference in fat excretion between normal and operated animals occurred. This suggests that fat absorption was essentially normal in these operated animals.

In the experiment summarized in Table II bile was collected for a 5-7 day period, followed by one, two, or three 5-7 periods of bile return on each diet. In all of these dogs fecal fat excretion was within normal limits (Table I) within 12 days after rejoining the cannulas. This means that steatorrhea in bile fistula dogs can be corrected by returning their own bile continuously.

Discussion. Fecal fat excretion was normal in operated dogs as long as 10 weeks after surgery (Table II). Thus, for studies requiring only periodic collection or sampling of bile this simple continuous return system might well keep animals in better condition than when no bile is recirculated, or when it is re-

TABLE I. Fecal Fat Excretion in Normal Dogs and in Dogs Circulating Their Bile through the Cannula System.

	Fecal excretion/day			
	Normal		Operated	
Food intake/day	No. of dogs	Total fat (g), mean ± S.D.	No. of dogs	Total fat (g), mean ± S.D.
			Without previous bile deprivation	
Control diet (300 g)*	9†	1.0 ± .9	5	1.0 ± 1.0
Plus 25 g oleic acid	4	1.0 ± .2	2	3.0 ± 1.2§
" 50 g " "	6	5.6 ± 2.7	2	6.4 ± 3.5
" 50 g triolein	7	1.7 ± .6	3	1.1 ± .5
			Following previous bile deprivation‡	
Control diet (300 g)*	9†	1.0 ± .9	4	1.0 ± .3
Plus 10 g oleic acid	6	1.7 ± 1.3	3	1.1 ± .4
" 25 g triolein	4	1.9 ± .8	3	.8 ± .2

* 10 g fat.
† These controls are the same series.
‡ These animals are the same as reported in Table II.
§ Group comparison: Operated *vs* normal dogs fed the same diet, $.02 < p < .05$.

TABLE II. Time Course for Correction of Steatorrhea in 6 Bile Fistula Dogs.

Fecal excretion, g total fat/day										
Control diet (10 g fat)				Plus 25 g triolein			Plus 10 g oleic acid			
	Bile return period*				Bile return period*			Bile return period*		
Bile fistula	1	2	3	Bile fistula	1	2	Bile fistula	1	2	3
11.5	1.0									
12.6	6.9	1.6	.8							
10.2	1.4			29.3	10.0	.7				
8.6	9.1	.9		25.0	5.0	1.0	15.0	3.0	1.3	1.8
				15.8	1.9	.8	13.0	4.1	1.0	.7
							21.7	11.6	1.4	

* Bile return period 1: 2- 7 days after reconnecting cannulas.
Idem 2: 7-12 *Idem* .
" 3: 12-17 " .

turned by stomach tube or other discontinuous technics. This system does not yet appear to be useful for more than 3 months. In many of the operated animals the gall bladder wall around the cannula eroded after this period, followed by leakage of bile and peritonitis. However, the animals exhibited surprising resistance to liver infections. While slight cholangitis was evident, histological sections revealed significant lesions of the liver parenchyma only in 3 dogs. In these 3 animals some evidence of hepatitis was present.

Summary. Eleven bile fistula dogs, with common bile duct sectioned, were permitted to recirculate their bile by periodically joining together polyvinyl cannulas fixed in the duodenum and gall badder and brought outside through stab incisions. In 6 of these dogs, fed varying amounts of triolein or oleic acid, steatorrhea was corrected completely within 12 days after rejoining the cannulas. In 5 other dogs fed similar diets, but not subjected to prior bile deprivation, fecal fat excretion was within normal limits for period up to 30 days. These results show that the steatorrhea of bile deprivation can be corrected by continuous bile return.

Technical assistance of Stanley Duberman and Thelma J. Dean is gratefully acknowledged.

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Effect of Dietary Calcium and Phosphorus Levels on Body Burdens of Ingested Radiostrontium.* (25434)

R. H. WASSERMAN AND C. L. COMAR
Dept. of Physiology, N. Y. State Veterinary College, Cornell University, Ithaca, N. Y.

Entry of radioactive strontium into the biosphere is one of the important consequences of fallout from nuclear explosions and much study has been given to interrelationships in the progression of fallout strontium and natural calcium through the food chain to human population. Details of comparative behavior of strontium and calcium in various species (under normal conditions and also during pregnancy and lactation) have been worked out and many of the findings con-

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TABLE I. Differences in Experimental Conditions of Two Reports on Influence of Dietary Calcium on Retention of Skeletal Sr* in the Rat.

Factors	Wasserman *et al.*(10)	Palmer *et al.*(11)
Sex of rat	♂	♀
Age of rat	Growing, young	Non-growing, mature
Ca supplement	$CaCO_3$	Ca lactate
P level	Ca/P maintained constant	P maintained constant
Sources of isotopes	Drinking water	Diet

firmed by independent methods(1-3). It is important to understand the extent to which dietary modifications can alter the levels of radiostrontium to be attained and to know how one can best predict future radiostrontium levels in various parts of the biosphere. Attention has recently been given to the effect of dietary calcium levels on retention of radiostrontium. In early studies the calcium carrier and radiostrontium usually were administered in a single dose and skeletal retention of radiostrontium measured shortly thereafter; little or no effect of calcium was seen under these conditions(4-7). Subsequently, double tracer technics with radiocalcium and radiostrontium were developed in comparison with lifetime feeding methods for assessment of calcium-strontium relationships(8,9). By use of these technics and also longer term studies, Wasserman *et al.*(10) showed that increased dietary level of calcium would indeed reduce proportionally the skeletal retention of radiostrontium in the growing rat; they also explained why the short-term retention of radiostrontium could not be used to predict steady-state body burdens. Palmer *et al.*(11) then published data to show that under certain experimental conditions an increased dietary level of calcium did not result in proportional decrease in retained radiostrontium in the mature rat. Table I summarizes the differences in experimental conditions used by the 2 groups of workers. The work reported here is an attempt to reconcile these apparently opposed findings, to explore further as to some of the factors that may be involved: comment is also offered on usefulness and limitations in consideration of strontium behavior in terms of calcium.

Methods. Details of procedure have been previously reported(10). Rats were initially placed on experimental diets for about 25 days; at this time the animals, while maintained on their respective diets, were transferred to individual cages and, for an additional 7 days, were given drinking water labeled with about 20 μc Ca^{45} and 10 μc Sr^{85}/liter as chlorides; these isotopes were essentially carrier-free. Water consumption and ingested radioactivity were estimated through use of graduated drinking tubes. In addition, body weight changes and feed consumption were determined during final 7 days. The rats were then killed and amount of Ca^{45} and Sr^{85} retained in both femurs determined by usual radiochemical procedures; the results are expressed as "percentage of ingested dose." The basal diet was as follows: ground yellow corn, 20%; dextrin, 29.2%; casein, 17.7%; brewer's yeast, 4.4%; Wesson oil, 2.9%; calcium-free salt mixture, 1.6%; Vit. mixture containing adequate levels of thiamine, riboflavin, pyridoxine, niacin, calcium pantothenate, choline, inositol, biotin, folic acid, Vit. B_{12}, p-aminobenzoic acid and Vit. A, D and E, 1%. The rest of the diet, 23.2%, was made up of the calcium salt (usually calcium lactate), K_2HPO_4 and cellulose fiber; the elevated levels of calcium and phosphorus salts replaced an equivalent weight of cellulose bulk.

Results. Table II is a summary of a study designed to compare all experimental differences except calcium/phosphate ratio and the source of tracers. Values for Observed Ratio (OR) compare reasonably well with previous data of Wasserman *et al.*(10) and indicate that major differences between the 2 groups of workers could not have been caused by sex, age, or type of supplement. In this study there appeared to be a small increase in OR value as a result of raising calcium and phosphorus level of the diet; this increase, however, was not large enough to have practical significance in relation to the effects of dietary calcium. In agreement with previous findings

TABLE II. Influence of Age and Sex on the Effect of Dietary Calcium and Phosphorus on the Femur Retention of Ca^{45} and Sr^{85} in the Rat.*

Group	Age	Sex	Dietary levels		Feed consumption (g/day)	Calcium intake (mg/day)	Final body wt (g)	Level in femur		OR†
			Ca (%)	P (%)				Ca^{45} (% ingested dose)	Sr^{85}	
I	young	♂	.5	.4	18.2	91	223	4.47 ±.12	1.00 ±.04	.22 ±.01
II	"	"	2.0	1.2	15.7	314	201	1.51 ±.03	.42 ±.01	.28 ±.03
III	"	♀	.5	.4	14.1	71	163	3.89 ±.14	.87 ±.06	.22 ±.01
IV	"	"	2.0	1.2	11.0	220	131	1.44 ±.06	.39 ±.03	.27 ±.01
V	mature	♂	.5	.4	21.5	108	434	.97 ±.07	.14 ±.01	.15 ±.01
VI	"	"	2.0	1.2	21.0	420	415	.41 ±.03	.09 ±.01	.21 ±.01
VII	"	♀	.5	.4	17.3	87	306	.86 ±.09	.13 ±.02	.16 ±.01
VIII	"	"	2.0	1.2	16.8	336	293	.34 ±.03	.07 ±.01	.19 ±.01

* Six rats/group; values represent mean ± stand. error of mean; Ca added as lactate salt and P as K_2HPO_4; animals on diets for 18 days prior to isotopes and 7 days on isotopes.

† $OR = \frac{Sr^*/Ca^* \text{ of bone}}{Sr^*/Ca^* \text{ of diet}}$.

(1), the younger animals showed somewhat less discrimination against Sr* relative to Ca* than did older animals (OR for young was about 0.22 compared with 0.15 for the mature).

Table III presents data from a study in which experimental conditions of Palmer *et al.* (11) were essentially duplicated, and the interaction of calcium and phosphorus further examined. In the mature rat, when dietary level of calcium alone was raised, OR value increased about 3-fold (from 0.13 to 0.38). This agrees with the results of Palmer *et al.* (11). It can also be noted that raising phosphorus alone (Group III *vs.* Group I) had no effect on relative retention. When both calcium and phosphorus were raised, OR value increased only from 0.13 to 0.20 (Group I *vs.* Group IV). These results also indicate that the technic of incorporation of tracers in drinking water gives the same results as incorporation in the diet.

Another study investigated calcium and phosphorus relationships in the growing rat, and, in addition, determined the effects of increased stable strontium on Ca^{45} and Sr^{85} retention (Table IV). It may first be noted that when dietary calcium alone was increased there was only a small increase in OR value (Group II *vs.* Group I) [as expected from earlier work(10) and Table II]; this is in contrast to behavior just described for mature animals. The effect of stable strontium is of particular interest. Rats in Group IV (0.5% Ca, 0.5% Sr, 0.4% P) showed a rachitic condition after 27 days on the diet, as indicated by (a) gross appearance of the femur, (b) decreased femur ash and (c) reduced deposition of both Ca^{45} and Sr^{85}. Simultaneous raising of phosphorus and strontium levels (Group V) overcame to a certain degree the rachitic effects of the strontium. It was of interest to observe that OR values for high strontium with and without additional phosphorus were about the same as for high calcium animals (Groups IV, V *vs.* Groups II, III). This means that stable strontium influenced radiocalcium behavior in much the same way that calcium itself did. Supplemental dietary strontium is thus less useful than calcium for

TABLE III. Interaction of Dietary Calcium and Phosphorus on Femur Retention of Ca^{45} and Sr^{85} in Mature Rat.*

Group	Dietary levels Ca (%)	P (%)	Feed consumption (g/day)	Calcium intake (mg/day)	Final body wt (g)	Level in femur Ca^{45} (% ingested dose)	Sr^{85} (% ingested dose)	OR†
I	.5	.4	20.4	102	256	1.03 ±.10	.13 ±.02	.13 ±.01
II	2.0	.4	19.8	396	262	.32 ±.04	.12 ±.01	.38 ±.04
III	.5	1.2	20.2	101	253	1.21 ±.08	.13 ±.02	.11 ±.01
IV	2.0	1.2	21.8	436	259	.34 ±.01	.07 ±.01	.20 ±.01

* Six rats/group; values represent mean ± stand. error of mean; Ca added as lactate and P as K_2HPO_4; animals on diets for 20 days prior to isotopes administration; 7 days on isotope feeding.

† $OR = \frac{Sr^*/Ca^* \text{ of bone}}{Sr^*/Ca^* \text{ of diet}}$.

reduction of Sr* burdens, because strontium at levels that would produce an appreciable effect also causes toxic side-effects; in addition, the discrimination by the intestinal barrier against strontium in favor of calcium would immediately reduce the parenteral concentration of strontium in contrast to the concentration of absorbed calcium.

Discussion. To predict the effect of dietary calcium or other dietary additives on the body burden of continuously ingested radiostrontium, it is most often necessary to compare retention of radiocalcium with that of radiostrontium. This is especially important when the experiment is done over a relatively short portion of the life span of the animal. Difficulties of interpretation mainly arise as a result of the adaptation of the animal to dietary changes. Although an increased ingestion of calcium may result in an immediate increase in utilization of calcium, the animal eventually will adapt such that the net retention of calcium is essentially independent of dietary calcium, within reasonable limits. By comparing Strontium-Calcium Observed Ratio (OR)† as defined by Comar *et al.*(9), the long term effect of dietary calcium can be predicted as follows: If an elevated dietary calcium does not alter the $OR_{diet\text{-}bone}$, it would then be apparent that, with time, the body burden of Sr* would be decreased inversely to increased calcium. Thus, the Observed Ratio (OR) can be of primary importance in prediction of long-term behavior.

A general comment is in order about variability that might be expected in the differential behavior of calcium and strontium in the mammal. The earliest observation had shown that strontium, in general, had a metabolic behavior similar to that of calcium. Substances that modified calcium behavior, also modified strontium behavior in a similar way (Vit. D, parathormone, lactose). Subsequent quantitative studies showed that there were differences

TABLE IV. Interaction of Dietary Calcium, Strontium and Phosphorus Levels on the Femur Retention of Ca^{45} and Sr^{85} in the Growing Rat.*

Group	Dietary levels Ca	Sr	P (%)	Feed consumption (g/day)	Alkaline earth intake (mg/day)	Final body wt (g)	Femur ash wt (mg)	Level in femur Ca^{45} (% of ingested dose)	Sr^{85} (% of ingested dose)	OR†
I	.5		.4	14.7	73	143	122	2.47 ±.16	.64 ±.06	.26 ±.01
II	1.0		.4	15.1	151	151	121	1.50 ±.06	.48 ±.02	.32 ±.02
III	1.0		.8	12.2	122	145	112	1.72 ±.07	.41 ±.04	.23 ±.01
IV	.5	.5	.4	14.5	145	103	47	.70 ±.05	.22 ±.02	.32 ±.01
V	.5	.5	.8	10.9	109	120	79	1.96 ±.10	.52 ±.04	.27 ±.01

* Six rats/group; values represent mean ± stand. error of the mean; calcium and strontium additions given as acetate salts, phosphorus as K_2HPO_4. Rats on diets for 14 days prior to isotopes and 7 days thereafter.

† $OR = \frac{Sr^*/Ca^* \text{ of bone}}{Sr^*/Ca^* \text{ of diet}}$.

† See Table II.

in behavior possibly related to the type of ion transport, *i.e.*, absorption from the gut, reabsorption in the tubules, transfer across the placenta, and secretion into milk(1).

Examination of data obtained by independent methods in many laboratories and many countries indicated that under usual dietary conditions OR values fall within a reasonably narrow range, with few indications of variation as high as a factor of two(2). It was also shown in early work that certain dietary modifications in the rat such as use of milk, lactose or lysine would decrease the discrimination against strontium relative to calcium by as much as a factor of two(1). In the very young rat, discrimination was also decreased (1). The present work shows that increased calcium with a high Ca/P ratio in the mature rat will also decrease discrimination, *i.e.*, cause an increased OR value.

With the realization that OR values can be varied by unusual conditions in individuals, it seems that the concept can be of value when properly used for the following purposes: (a) estimation of levels of radiostrontium in local areas of bone since the Sr*/Ca of newly forming bone is related to Sr*/Ca of the contemporary diet, (b) estimation of body burdens and local concentrations to be attained in populations from knowledge or estimates of Sr*/Ca ratios to be attained in food sources, (c) estimation of effects of life-time dietary changes from short-term experiments.

It is emphasized that the concepts of Sr-Ca action should be used where they can provide advantages; likewise, data must also be obtained and reported concurrently on absolute levels of calcium and radiostrontium in the biosphere so that these data can also be used as necessary. Such data are needed when it is required to sum the various constituents of a diet to give the Sr-Ca ratio in the total diet.

The reported differences in the effect of dietary levels on radiostrontium behavior(10, 11), can be explained as follows: In young, growing rats increase of dietary calcium, with or without proportionate increases of dietary phosphorus, caused little or no change in relative retention of calcium and strontium. In the mature rat, an increase of dietary calcium alone caused about a 3-fold decreased retention of radiocalcium as compared to radiostrontium retention; when dietary phosphorus was also increased the effects on Ca* and Sr* were more nearly similar. The reasons for this behavior must be related to the mechanisms of handling the 2 elements which probably differ with age of the animal. These aspects are now under active study.

It must be pointed out that these observations should not be used as a basis for recommendation of changes in human or animal dietaries. Consideration would have to be given to such matters as the extent to which the dietary modification proposed would actually change the body burden of radiostrontium under the given conditions. Also, it would have to be determined whether or not the long term feeding of increased calcium would have deleterious effects on a population or individual basis that would outweigh the benefit to be gained by any reduction in the radiostrontium burden.

Summary. It is emphasized that long term effects of dietary constituents on radiostrontium must be determined either by long term experiments or predicted from double tracer technics. Previous reports in disagreement as to the effect of increased dietary calcium on radiostrontium have been reconciled with predictions as follows: (a) in immature rats, elevated dietary calcium levels (within physiological ranges) with or without increased phosphorus levels would almost proportionately reduce the body burden of dietary radiostrontium, (b) in mature rats, elevated dietary calcium levels alone would not proportionately reduce the radiostrontium and (c) in mature rats, simultaneous increases in dietary calcium and phosphorus levels would to some degree reduce the ultimate body burden of radiostrontium.

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Erythropoietic Factor in Kidney Tissue of Anemic Dogs.* (25435)

JEAN-PIERRE NAETS† (Introduced by John H. Lawrence)
Donner Lab., and Lawrence Radiation Lab., University of California, Berkeley

In previous communications it was shown that bilateral nephrectomy abolished erythropoiesis in the dog(1) and that ureter ligation did not impair this function despite the same degree of uremia and malnutrition(2). In other studies an erythropoietic factor was demonstrated in plasma and urines of bled dogs(3) and disappearance of this factor was shown to occur after bilateral nephrectomy (4). These results agree with experiments by Jacobson *et al.*(5) on the rat and suggest that the kidney is the source of an erythropoietic stimulating factor. Several attempts to point out the erythropoietic factor in acidified boiled filtrates from kidney and other organs(6,7,8) have been unsuccessful. This study was an attempt to demonstrate erythropoietic stimulating activity in kidney homogenates.

Materials and methods. Fifteen mongrel dogs weighing between 13 and 25 kg were used. Three were acutely bled to a hematocrit of 14—9% in 6 hours at which time they were bled to death by femoral puncture. Twelve were made anemic (hematocrit 19—5%) by bleeding for 3 to 5 days. In this group 6 dogs were unilaterally nephrectomized 10 to 20 days before bleedings; the removed normal kidneys were used as normal controls. Blood volume was maintained by Dextran infusion as described elsewhere(3). Some dogs died from anemia and the kidneys were taken immediately after death; in other experiments the kidneys were removed under nembutal anesthesia before the animal's death. In 8 experiments the liver was also removed and in 5 the spleen. Removed organs were kept frozen until used. Kidney, liver and spleen tissue were ground with saline in a Waring Blendor for 3 to 5 minutes; saline volume added 150% of the weight of each organ; the tissue suspension was filtered through gauze and injected subcutaneously into rats without further treatment. Erythropoietic activity of organ suspensions was measured by Fe^{59} red cell incorporation assay on starved rats(9), as described in previous paper(3). Recipient rats received 3 ml of organ suspension subcutaneously daily for 2 successive days before Fe^{59} injection. Four to 10 rats were used for each determination. Controls consisted of uninjected rats and groups receiving normal kidney suspension, "anemic" liver suspension and "anemic" spleen suspension. After organ emulsion injections all rats developed abscesses at the site of injection and of the 253 rats used, 26 died from the toxicity of the material injected; no difference in toxicity was noticed for the extracts from different tissues. Hematocrits were measured in Wintrobe tubes by centrifugation at 3,000 rpm for 30 minutes. Hemoglobin was deter-

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† Charles Pecher post-doctoral Fellow at University of California on Fulbright Travel Grant. Present address: Brugmann University Hospital, University of Brussels.

TABLE I. Effect of Ground Tissue Injection on Fe^{59} Uptake into Red Cells of Starved Rats.

Donor dogs			% Fe^{59} uptake into red cells of recipient rats					
No.	Hematocrit, %	Hemoglobin, %	Uninj. control	Normal kidney inj.	"Anemic" kidney inj.	"Anemic" liver inj.	"Anemic" spleen inj.	Duration of bleeding
34	5	2.2	5.6 ± 1.4 (6)†	6.0 ± 2.8 (5)	9.9 ± 4.7 (5)			4 days
38	14		4.6 ± .8 (6)	8.0 ± 4.2 (5)	16.7 ± 7.9 (9)			6 hr
				4.7 ± 3.3 (7)				
40	9		4.6 ± .3 (10)	8.3 ± 1.8 (8)	12.2 ± 5.8 (8)	8.3 ± 4.6 (8)		6 "
41	16	4.8*						
45	11	3.6	5.1 ± 2.8 (9)	7.1 ± 2.7 (7)	20.3 ± 6.1 (8)		6.5 ± 2.1 (4)	3 days
42	19	5.8	3.8 ± .7 (9)	14.6 ± 4.0 (8)	11.4 ± 3.5 (7)			3 "
46	11	3.2	*Idem*	*Idem*	20.5 ±11.6 (8)	10.6 ± 4.1 (8)	20.1 ± 8.9 (8)	4 "
44	11	5.2	3.3 ± .6 (10)	7.6 ± 2.8 (6)	10.9 ± 3.3 (8)	11.4 ± .3 (8)		6 hr
47	14	5.8	5.3 ± 1.9 (10)	9.3 ± 2.1 (8)	11.5 ± 7.0 (6)	11.7 ± 5.8 (7)	8.5 ± 3.9 (7)	4 days
				13.6 ± 5.8 (8)				
48	9	3.2	*Idem*	*Idem*	14.2 ± 7.1 (6)			5 "
50	8	3.8	4.9 ± .6 (10)	9.6 ± 4.8 (8)	11.3 ± 7.1 (7)			5 "
57	8	3.4	*Idem*	*Idem*	19.8 ± 5.2 (7)	10.3 ± 3.8 (7)		5 "
55	7	2.0	4.8 ± .6 (7)	11.4 ± 2.3 (6)	13.5 ± 5.1 (8)	8.6 ± 3.7 (6)		5 "
61	8	3.8	4.9 ± .7 (6)		17.5 ± 3.3 (4)			4 "
64	8		*Idem*			14.0 ± 3.9 (6)	10.0 ± 5.4 (7)	4 "
65	8	2.8	"		20.1 ± 3.9 (4)	9.7 ± 5.8 (5)	4.5 ± 1.6 (4)	3 "
	Mean		4.7 ± .9	9.1 ± 4.5	15.0 ± 7.2	10.6 ± 4.4	9.9 ± 7.9	
	t			4.05		2.92	2.4	
	p			<0.001		<0.01	0.02 < p < 0.05	

* Livers from dogs No. 41 and 45 were combined, and so were the kidneys.
† Stand. dev.
Figures in parentheses indicate No. of rats in each assay.

TABLE II. $Iron^{59}$ Uptake into Red Cells of Starved Rats Compared with Iron Plasma Level of the Same Blood Samples.

	Normal kidney		"Anemic" kidney		"Anemic" liver		"Anemic" spleen		Control uninj.	
Donor dog No.	Fe plasma level (pool values), μg %	Fe^{59} uptake, %	Fe plasma level (pool values), μg %	Fe^{59} uptake, %	Fe plasma level (pool values), μg %	Fe^{59} uptake, %	Fe plasma level (pool values), μg %	Fe^{59} uptake, %	Fe plasma level (pool values), μg %	Fe^{59} uptake, %
47	142	9.3 (8)	126	11.5 (6)	112	11.7 (7)	164	8.5 (7)		
48	93	13.6 (8)	100	14.2 (6)						
55	62	11.4 (6)	62	13.5 (8)	39	8.6 (6)				
57	104	9.6 (8)	95	19.8 (7)	119	10.3 (7)				
50			93	11.3 (7)						
61			178	17.5 (4)						
64					152	9.7 (5)	158	10.0 (7)	276	4.9 (6)
65			129	20.1 (4)	95	14.0 (6)	138	4.5 (4)		
Avg	100	11.0	115	15.9	103	10.9	153	7.7	280 ± 38*	4.7†

* Avg of 10 individual measurements; other iron plasma assays are made with pooled plasma.
† Mean of 83 control uninj. rats used.
Figures in parentheses indicate No. of rats used in each assay.

mined by the method of Crosby (Cyanmethemoglobin method)(10). Plasma iron level determinations were made by technic of Peters *et al.*(11).

Results are summarized in Table I. Rats injected with "anemic" kidney tissue showed larger uptake of Fe^{59} into red cells than those injected with normal kidney, "anemic" liver and "anemic" spleen. After "anemic" kidney injection the uptake was 15.0% (from 9.9 to 20.5%) while after normal kidney, "anemic" liver or "anemic" spleen injections it averaged 9.1% (4.7 to 14.6%), 10.6% (8.3 to 14.0%), and 9.9% (4.5 to 20.1%) respectively.

The significance of the difference in Fe^{59} uptake, between groups of rats injected with "anemic" kidney emulsion and rats receiving other tissue emulsions, is indicated at the bottom of Table I. After only 6 hours bleeding (dogs No. 38, 40 and 44), "anemic" kidney suspension induced increased Fe^{59} uptake. Twenty-six control rats were injected with normal kidney and compared with 25 rats receiving "anemic" kidney from dogs No. 38, 40 and 44 (Table I); iron uptake was 7.1% $\pm$ 3.1 after injection of normal kidney and 13.4% $\pm$ 6.6 after "acute anemic" kidney administration. The difference between these two values (anemia of 6 hours duration) is significant.

Discussion. It can be seen from the Table that Fe^{59} uptake is similar after injection of normal kidney, "anemic" liver and "anemic" spleen but higher than iron uptake observed in uninjected starved rats. This increased iron uptake seems to be nonspecific and could be related to the caloric content of the administered material(9). Nevertheless the caloric content of 3 ml of organ suspension (which corresponds to 1.2 g wet tissue) is low, about 2 calories. It is unlikely that "anemic" kidney is more calorigenic than normal kidney or "anemic" liver.

The possibility exists that injected foreign proteins lower the plasma iron level in the recipient rats, thus increasing the Fe^{59} uptake into red cells(12). In order to test this possibility, plasma iron level was measured in pooled rat plasma obtained by centrifugation of blood taken for Fe^{59} uptake measurements. The results are presented in Table II. After tissue injections the plasma iron level was lower than in uninjected starved rats. This lower plasma iron level, together with the few calories injected could explain the higher Fe^{59} uptake observed after injection of organ suspensions. On the other hand, Table II demonstrates that injected anemic kidney tissue did not decrease plasma iron level of the recipient rats more than normal kidney and "anemic" liver injections. This suggests that the higher iron uptake obtained after anemic kidney injection was related to an erythropoietic factor present in the tissue. This factor does not come from the anemic plasma contained in the kidney since even if very active the amount of contained plasma was too small to induce increased Fe^{59} uptake. Injections of 2 to 6 cc of anemic dog plasma are required to induce increased iron uptake in starved rats(3,4); furthermore, "anemic" liver and "anemic" spleen contain about the same amount of active plasma as "anemic" kidney.

Although erythropoietic activity of the kidney of anemic dogs was not sufficiently greater than that of other tissues to demonstrate conclusively the importance of the kidney in erythropoietin production, it was sufficient to indicate that with adequate methods of isolating and concentrating the activity from crude homogenates a conclusive demonstration might be possible. Fractionation procedures are being investigated.

Summary. Homogenates of kidney, liver and spleen tissue from normal and anemic dogs were assayed for erythropoietic activity by their effect on Fe^{59} red cell incorporation in starved rats. A slightly higher incorporation resulted from administration of an extract of anemic kidney than from extract of normal kidney or anemic liver or anemic spleen. This difference was sufficient to suggest the importance of developing methods for concentrating and purifying the erythropoietic activity of tissue homogenates to help clarify the role of kidney in production of erythropoietin. Methods for fractionation and concentration are being investigated.

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Further Studies on Effects of Tranquilizing Drugs on Mammary Involution in the Rat.* (25436)

G. K. BENSON (Introduced by R. Gaunt)

Nat. Inst. for Research in Dairying, Shinfield, Reading, Berkshire, England

Oxytocin administration inhibits mammary involution in rats after removal of the litter at fourth day of lactation, and it was suggested that oxytocin elicits prolactin release under these conditions(1,2). Later, following reports of its lactogenic effects in several species (3-7), it was shown that reserpine resembles oxytocin in showing a striking effect in retarding mammary involution in the rat(8). It was of interest to study effects on mammary involution of other tranquilizing drugs, and the present paper describes experiments carried out with chlorpromazine, reported to cause galactorrhoea in women(9,10), and syrosingopine (Singoserp), an analogue of reserpine possessing hypotensive properties similar to those of reserpine, but with a considerably reduced central sedative action(11,25). To throw light on the mechanism of action of reserpine, the effects of hypophysectomy and of adrenalectomy on reserpine-induced parenchyma maintenance have been investigated. Further, in view of claims that the pharmacological action of reserpine may be due to altering the serotonin-binding capacity of brain cells(12,13), the effects of serotonin on mammary involution have been studied; and since reserpine has been reported to release histamine from blood platelets(14), the effect of this substance on mammary involution was also investigated.

Materials and methods. Hooded Norway rats were used, 2-3 months old, undergoing first lactation. Stock colony diet(15) was fed *ad lib.;* daily food and water consumption was recorded. After parturition all litters were reduced to 9 young on first day of lactation, and to 8 on second day. Litters were weighed daily to check lactational performance of the mother, and mothers were also weighed daily as in previous experiments. Litters were removed on 4th day of lactation when treatment began and when operations, if any, were carried out. Substances administered during treatments were a) Reserpine (Serpasil†), b) Syrosingopine (Singoserp‡), c) Chlorpromazine (Largactil, May & Baker Ltd.), d) 9 α fluorocortisol (FC) supplied as acetate by E. R. Squibb & Sons, N. J.; this was administered in suspension in 0.5 ml carboxy-methylcellulose (CMC) solution, e) Serotonin, supplied as creatinine sulphate by May & Baker Ltd., f) Histamine, supplied as acid phosphate by British Drug Houses Ltd. Full details of experimental groups, dosages,

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etc. are included in Table I. At end of treatment period, which except where otherwise stated lasted 9 days, mammary tissue was studied histologically, alveolar maintenance estimated by determination of percentage parenchyma(2). Various other organs were removed, weighed, and preserved for histology. Where appropriate, brain and pituitary region, suitably trimmed, was sectioned after decalcification, and checked for pituitary remnants.

Results. Reserpine and Syrosingopine. In this experiment both drugs were made in citric acid-glycol vehicle, as follows:—citric acid anhydrous 0.25%; benzyl alcohol 2%; polyethylene glycol 10%; distilled water q.s. 100%. Stock solutions were diluted to enable necessary quantities to be injected in 0.1 ml of the vehicle.

Mammary gland involution was retarded to a similar degree by all treatments except lower dose of syrosingopine, where maintenance was less well-marked (Table I). The control group showed some maintenance, quantitatively similar to that previously found for intact animals receiving ACTH(16,17), and may therefore be due to a non-specific stress effect of the vehicle. As in previous experiments(8) a significant decrease in uterine weight was observed as a result of reserpine treatment, though not after syrosingopine treatment (Table I), and histological examination indicated that in the control group, weaning was followed by return to normal ovarian cyclic activity. This finding was borne out by absence of mucification in the control vaginae; the vaginae of 9 of reserpine-treated animals and 7 of syrosingopine-treated animals were mucified. As before(8) thymus weights were depressed by reserpine at both dose levels, particularly the higher, and also, by syrosingopine at higher dosage. Adrenal weights were increased by higher dose of reserpine but surprisingly a slight decrease in mean adrenal weight was observed in the lower dosage group. Syrosingopine had no effect.

Depression of body weight previously noted after administration of 100 μg reserpine daily (8) was again observed, but all other treatments resulted in significant gains in body weight. The higher dose of reserpine was obviously in the toxic range, since animals so treated exhibited severe diarrhoea and deterioration in coat condition. Syrosingopine did not produce effects of this nature.

Chlorpromazine. Dosages were higher than those reported by Sulman & Winnik(18) to depress cyclic ovarian activity in rats. However, chlorpromazine was much less effective than reserpine in retarding mammary involution, though at the higher dose level it was somewhat more potent than the lower dose level of syrosingopine (Table I). Ovaries of the treated groups were significantly lighter than those of control group, but no significant differences in uterine weight were apparent. Histological examination showed evidence of ovarian cyclic activity in some treated animals, as in all control animals. Treated animals not showing resumption of ovarian cyclic activity had mucified vaginae. Ovaries of the control group contained many very large corpora lutea. Little influence on adrenal and thymus weights was apparent, though the lower dose level appeared to have depressed adrenal weight below the control value. The depressive effect on body weight after reserpine treatment (Table I) was not a feature of chlorpromazine administration.

Reserpine in hypophysectomized animals. All hypophysectomized and sham operated animals lost weight rapidly during treatment period. By the 6th day after operation, 3 hypophysectomized animals were in a parlous condition, and were therefore killed, 3 controls were killed on equivalent day to match them. The other 2 hypophysectomized animals survived to the 13th day after parturition, when they and remaining controls were killed. No residual fragments of anterior pituitary were detected in hypophysectomized animals in sections of brain.

Involution of mammary tissue in hypophysectomized animals was quite clearly unaffected by reserpine. Even in rats killed on 10th day of lactation, marked involution had occurred (Table I). In contrast, administration of reserpine to the control group again sustained integrity of the parenchyma to a marked degree. In these sham-operated animals percentage parenchyma values indicated no fall-off in degree of maintenance between 10th and 13th day of lactation. In hypophy-

TABLE I. Effects of Various Treatments on Percentage Parenchyma, Body Weight, Organ Weights and Food and Water Consumption in Lactating Rats Following Removal of Litters on 4th Day of Lactation, When Treatments Commenced.

Treatment	Daily dosage and route of admin.§	Day of lactation on which animals were killed	No. of animals	Mean % parenchyma	Mean % change in body wt over treatment period	Mean daily food consumption over treatment period (g)	Mean daily water intake over treatment period (ml)	Mean wt (mg)/100 g body wt				
								Uterus	Ovaries	Thyroids	Thymus	Adrenals
Controls, vehicle only	0.1 ml s.c.	13	5	30.5 ± 1.6	− 2.1 ± .6	10.6 ± .5	19.3 ± 1.4	195.7 ± 15.0	30.0 ± 2.7	14.4 ± 1.7	127.2 ± 9.7	32.5 ± 1.3
Reserpine	100 μg "	13	5	53.6 ± 6.0†	−11.6 ± 2.4†	10.6 ± .7	20.0 ± 1.1	109.8 ± 9.0‡	31.4 ± 2.4	12.4 ± .9	52.5 ± 2.1‡	41.2 ± 2.2†
"	50 μg "	13	5	49.7 ± 4.3‡	+ 7.2 ± .9‡	16.6 ± 2.7*	27.9 ± 1.2‡	129.0 ± 15.0†	26.1 ± 1.5	13.4 ± 1.6	97.6 ± 5.8*	27.1 ± 1.7*
Syrosingopine	100 μg "	13	5	57.6 ± 2.6‡	+ 7.6 ± 4.0*	12.4 ± .9	21.4 ± 3.3	160.8 ± 27.1	23.6 ± 1.1*	12.1 ± 1.6	85.9 ± 12.2*	30.8 ± 1.9
"	50 μg "	13	5	33.3 ± 7.5	+ 9.6 ± 2.8‡	13.9 ± .6†	27.8 ± .8‡	186.9 ± 19.8	28.2 ± 1.2	12.2 ± .3	106.4 ± 11.6	28.2 ± .6
Saline	.5 ml "	13	6	24.9 ± 5.7	− 1.1 ± .4	13.5 ± .5	21.3 ± 2.2	190.9 ± 15.4	47.7 ± 4.0	13.9 ± .7	133.5 ± 7.2	34.1 ± 1.7
Chlorpromazine	2.5 mg "	13	7	25.5 ± 5.1	− 0.8 ± .4	16.0 ± .7‡	19.9 ± .9	182.2 ± 9.9	31.0 ± 1.1‡	13.8 ± .4	139.3 ± 7.2	28.3 ± 1.9*
"	5.0 mg "	13	7	41.8 ± 7.1*	− 3.6 ± .6‡	15.4 ± .7†	19.6 ± 1.4	166.3 ± 18.3	33.0 ± 1.7†	13.7 ± .9	136.0 ± 15.4	30.8 ± 1.6
Hypophysectomy + reserpine	100 μg "	10	3	24.8 ± 4.3†	−19.1 ± .9*	7.5 ± .7	39.2 ± 6.6	84.3 ± 6.2†	31.2 ± 2.5	10.9 ± .4	83.0 ± 3.1	24.1 ± 1.6†
		13	2	21.6 / 25.6 } 23.6	−24.5 / −26.9 } 25.7	8.9 / 8.8 } 8.8	44.8 / 37.1 } 40.9	82.6 / 62.7 } 72.6	23.4 / 26.7 } 25.0	9.4 / 7.1 } 8.2	116.4 / 74.7 } 95.5	20.4 / 16.9 } 18.6
Sham-operation + reserpine	100 μg "	10	3	55.7 ± 7.7	−10.1 ± 3.1	12.3 ± 2.6	26.9 ± 2.5	110.4 ± 4.9	27.9 ± 1.7	11.0 ± .8	77.6 ± 18.7	33.4 ± 2.0
		13	3	70.3 ± 8.9	−26.5 ± 1.1	8.7 ± 1.1	16.7 ± 2.0	109.8 ± 2.6	24.9 ± 1.9	10.0 ± .6	56.4 ± 9.9	37.9 ± 3.1
Adrx. + saline	.5 ml "	9	4	37.8 ± 5.7	−15.0 ± 2.7	9.3 ± 1.2	36.0 ± 4.0	175.1 ± 40.4	33.3 ± 2.8	12.1 ± .9	115.4 ± 20.3	
		13	2	12.2 / 19.5 } 15.8	−12.3 / −15.8 } −14.0	8.1 / 8.7 } 8.4	33.7 / 39.7 } 36.7	167.9 / 130.2 } 149.0	38.9 / 37.6 } 38.2	11.3 / 11.4 } 11.3	128.5 / 210.2 } 169.3	
Adrx. + reserpine	100 μg "	9	4	50.7 ± 2.2*	− 7.0 ± 2.4*	10.4 ± 1.1	20.0 ± 1.5†	104.9 ± 10.1	30.1 ± 3.5	12.7 ± 1.3	96.4 ± 25.5	
		13	2	29.5 / 34.3 } 31.9	−25.8 / −24.1 } −24.9	9.5 / 10.1 } 9.8	14.8 / 18.8 } 16.8	217.9 / 115.7 } 166.8	37.9 / 34.1 } 36.0	9.7 / 14.8 } 12.2	96.4 / 132.7 } 114.5	
Adrx. + CMC sol.	.5 ml "	13	6	20.0 ± 3.3	−15.2 ± 1.2	8.3 ± .4	26.7 ± .4	195.2 ± 13.1	39.3 ± 1.2	15.4 ± .8	162.1 ± 6.9	
Adrx. + reserpine + FC	100 μg " 100 μg "	13	5	65.3 ± 5.3‡	−29.7 ± .7‡	6.7 ± .2*	10.3 ± .6‡	110.2 ± 4.8‡	33.8 ± 1.6†	14.8 ± .6	52.8 ± 3.7‡	
Saline 3×	0.5 ml i.p.	13	5	25.7 ± 4.4	+ 2.3 ± 3.0	12.1 ± .5	25.7 ± 1.0	226.5 ± 26.7	32.3 ± 2.6	14.1 ± 2.2	120.1 ± 13.8	28.6 ± 2.3
Serotonin "	250 μg "	13	5	24.8 ± 1.9	− 0.5 ± 4.1	12.6 ± 1.0	25.5 ± 1.7	218.1 ± 25.6	36.2 ± 1.4	14.6 ± 1.0	105.6 ± 7.7	34.5 ± 1.4*
Histamine "	1.0 mg "	13	5	31.7 ± 7.3	+ 4.7 ± 2.5	11.3 ± .7	22.5 ± 2.0	198.1 ± 31.5	30.6 ± 2.6	12.8 ± 1.0	103.6 ± 15.0	27.0 ± 1.2

Values of P for differences between treatment and control means: * $.02 < P < .05$; † $.001 < P < .01$; ‡ $P < .001$.

§ s.c. = subcutaneous; i.p. = intraperitoneal.

sectomized animals, reduced organ weights presumably reflected absence of trophic hormones. In controls, reserpine greatly depressed thymus weights, and some elevation of adrenal weight was apparent. Depressed ovarian and uterine weights in these sham-operated animals expressed lack of cyclic function, borne out by atrophic appearance of these organs when examined histologically. Vaginae of several sham-operated groups were mucified. Over the 6-day period, body weight of hypophysectomized animals decreased at significantly higher rate than did controls, but at 13 days, loss of body weight in both groups was about equal, and similar to that previously reported after reserpine administration(8). Food consumption did not vary significantly over the 6- or 9-day period and was low throughout both groups. Water consumption was generally higher in hypophysectomized animals.

Reserpine in adrenalectomized animals. Dosage of FC in this experiment has previously been shown to give a considerable degree of lactation maintenance in adrenalectomized rats(19). Combined effects of the operation and reserpine treatment resulted in rapid loss of condition in a number of the group receiving reserpine alone, confirming findings of Gaunt *et al.*(20) and 4 of these animals were either dead at 9th day of lactation or were killed because of their condition. Four of control saline-injected group were therefore killed on 9th day of lactation, thus leaving 2 animals in each of these 2 groups, all of which survived to 13th day (Table I). All animals receiving corticoid in addition to reserpine and controls receiving CMC solution survived to 13th day.

Over 5-day and 9-day treatment reserpine alone had some effect in maintaining the mammary gland in absence of adrenals (Table I), though degree of maintenance was by no means as marked as in intact animals already described(8). However, when FC was administered in addition to reserpine a very marked degree of maintenance was achieved (Table I). Little significant information can be derived from organ weights which differed widely within groups. Histological examination of uteri, ovaries and vaginae revealed little sign of cyclic activity within reserpine treated groups, vaginae generally being mucified. In control groups, however, some uteri and ovaries showed clear evidence of a return to normal ovarian cycles following weaning.

Serotonin. The dosage used (250 μg 3 $\times$ daily) was referred to the 5 H-T base, and each dose was contained in 0.5 ml 0.9% saline. No marked effects were observed with respect to maintenance of mammary parenchyma, either qualitatively or quantitatively (Table I). There were no significant effects on organ weights, except for an increase in adrenal weight after serotonin administration. Body weights and food and water intakes were not affected.

Histamine. Each dose (1 mg 3 $\times$ daily) was contained in 0.5 ml saline. No effects of mammary involution were observed (Table I), and there were no effects on organ weights, body weight, or food and water intakes.

Discussion. That reserpine retards mammary involution in rats(8) has been amply confirmed in this experiment. This finding, together with histological studies on uteri and vaginae, supports the view, first suggested by lactogenic responses(3-7, 21), that reserpine released prolactin. Syrosingopine was less effective in retarding mammary involution than was reserpine. In view of the reduced central sedative action of this analogue the finding lends support to the theory that prolactin release by these drugs is achieved by suppression of a center in the hypothalamus which normally inhibits prolactin release.

It is interesting that the retarding action of chlorpromazine on mammary involution was less than one-fiftieth of that of reserpine on a weight for weight basis, although dosages used were larger than those previously reported to depress anterior-pituitary function in rats (18).

The possibility that the effects on mammary involution are due to a direct effect of reserpine on the mammary gland receives no support from the experiment with hypophysectomized animals, retardation of mammary involution being obtained only in sham-operated rats. This finding, together with supplementary observations on uteri and vaginae,

supports the inference that the pituitary gland is involved, as in experiments with oxytocin (2). However, whether or not this is due to a "permissive action" of anterior-pituitary hormones cannot be determined on the basis of this work.

Unlike hypophysectomy, adrenalectomy does not totally impair the effect of reserpine on retardation of mammary involution, though it markedly diminishes it, the full reserpine effect being restored by corticoid administration. Reserpine appears to stimulate ACTH release, indicated here by changes in thymus and adrenal weight, and also reported elsewhere(20,22). It might be thought that its effects in these experiments could be attributed to ACTH release in view of the established importance of this hormone in lactation(23). However, both ACTH and adrenal corticoids, including FC, have previously been shown in similar experiments to possess only a relatively slight effect in retarding mammary involution in intact animals(16, 17,24). It therefore seems unlikely that the whole of the reserpine effect can be attributed to stress-induced release of ACTH. The comparative failure of reserpine to retard mammary involution in adrenalectomized animals, unless corticoids are administered, may be explained in terms similar to those suggested in connection with oxytocin-induced release of prolactin in adrenalectomized animals(17), *i.e.*, in absence of adequate levels of adrenal steroids, secretion of prolactin by the anterior lobe is reduced.

The general lack of effect of syrosingopine on adrenal and thymus weights in these experiments confirms the findings of Chart, Renzi & Gaunt (personal communication) who also observed no adrenal weight increase following chronic administration of this substance, and is in harmony with their observations that reserpine is 5 or more times as potent as syrosingopine in causing adrenal ascorbic acid depletion. The results reported here do not confirm the findings of Meites *et al.*(26) that serotonin retarded mammary involution in the rat, and they lend no support to the possibility that action of reserpine in retarding mammary involution may be mediated by serotonin or histamine. These findings conform with those of Sawyer(5) who observed no mammary activation following injections of serotonin into the cerebral ventricles of suitably prepared rabbits. Reserpine was, however, effective in stimulating mammary growth and secretion when administered in this way.

In general, our findings are in keeping with the suggestion discussed elsewhere(27), that the effects of reserpine on the mammary gland (both lactogenic and involution-retarding effects) are due to removal of a hypothalamic inhibition of prolactin release.

Summary. 1. In rats, following weaning of litters on 4th day of lactation, administration of syrosingopine (50 μg daily) for 9 days had much less effect than the same dose of reserpine on retardation of mammary involution, but was similar in effect at a higher dose level (100 μg daily). 2. Chlorpromazine (2.5 mg and 5 mg daily) also had a lesser retarding effect on mammary involution than reserpine. 3. No noticeable effects on mammary involution were observed after administration of serotonin (250 μg 3 $\times$ daily) or histamine (1 mg 3 $\times$ daily). 4. Reserpine (100 μg daily) had no effect on mammary involution in hypophysectomized animals. 5. In adrenalectomized animals some involution-retarding effect of reserpine (100 μg daily) was still apparent and was greatly enhanced by addition of 9α fluorocortisol. 6. Reserpine, at higher dose level, depressed food consumption and body weight, and retarded return of normal cyclic function after weaning as did the higher dose of syrosingopine to a lesser degree. Marked effects on thymus and adrenal weights after reserpine administration indicated that ACTH secretion is stimulated by the drug, but neither syrosingopine nor chlorpromazine treatment resulted in any apparent stimulation of ACTH release. 7. The significance of these findings in relation to a possible hypothalamic inhibition of prolactin release is discussed.

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Indirect Evidence of Synthesis of Norepinephrine from 3-Hydroxy-Tyramine-1-C^{14} *in vivo*.* (25437)

M. GOLDSTEIN, A. J. FRIEDHOFF, C. SIMMONS AND N. N. PROCHOROFF
(Introduced by N. Nelson)
Dept. of Psychiatry and Neurology, Psychopharmacology Research Unit, N. Y. U. College of Medicine

Considerable evidence is available that 3-hydroxytyramine is, *in vitro,* a precursor of norepinephrine(1,2,3). However, little evidence is available about the formation of norepinephrine from 3-hydroxytyramine in the intact animal. The low rate at which 3-hydroxytyramine is utilized for conversion into norepinephrine, as well as the high rate of metabolism of 3-hydroxytyramine and norepinephrine *in vivo* makes it difficult to demonstrate this reaction directly in the intact animal. Since Axelrod(4) has shown that the main metabolite of norepinephrine is 3-methoxy-norepinephrine, it occurred to us that conversion of 3-hydroxytyramine to norepinephrine could be demonstrated *in vivo* indirectly by isolation of the end product of biosynthesis, namely, 3-methoxynorepinephrine. Also, since various kinds of stresses have been reported to increase excretion of norepinephrine, it seemed likely that excretion of 3-methoxynorepinephrine would be affected under these conditions.

Materials. Iproniazid phosphate was kindly supplied by Hoffmann-La Roche; 3-hydroxytyramine-1-C^{14} was obtained from New England Nuclear Corp.; 3-methoxytyramine-1-C^{14} was isolated from urine of rats treated with iproniazid; 3-methoxynorepinephrine, 3-methoxyepinephrine and 3-methoxytyramine were kindly supplied by Sterling-Winthrop

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Research Inst.; and hog kidney acylase was obtained from Nutritional Biochemicals Corp.

Methods. Groups of 6 rats were treated with 100 mg/kg of iproniazid and 100 μg of 3-hydroxytyramine-1-C^{14}, injected daily. Urine was collected for 24 hours, and samples were hydrolyzed at pH 2 by refluxing them under nitrogen for 30 minutes. After cooling to room temperature, the urine was extracted 4 times with ethyl acetate. The organic layer contained all of the acid metabolites of 3-hydroxytyramine, which were further separated by paper chromatography(5). The aqueous layer was adjusted to pH 4, and the amines were acetylated as described previously(6). The acetylated amines were extracted into methylene chloride, and aliquots of the concentrated extracts were applied for chromatographic separation in the "C" solvent system of Bush(7). Dry chromatograms were then scanned in a calibrated counting chamber, so as to locate all radioactive zones. Material in the radioactive zones was eluted with methyl alcohol and concentrated under nitrogen. Aliquots of concentrated eluates were dried on planchets, and radioactivity measurements were made with Tracerlab SC-16 windowless flow counter on duplicate samples for a time to assure an accuracy of ± 5%. *Deacetylation.* Dry samples of the acetylated catecholamines or methoxycatecholamines were dissolved in 1 to 2 ml water, and 50 to 100 mg of hog kidney acylase was added. The mixture was incubated 3 hours at 37°C, with air as the gas phase. At end of incubation, *O*-acetyl groups were removed and *N*-acetyl derivatives were extracted from the aqueous phase into ethyl acetate. The combined ethyl acetate extract was concentrated *in vacuo,* and aliquots were chromatographed in the "C" system of Bush for 24 hours, then in amyl alcohol-water for 12 hours, or in a chloroform-formamide solvent system overnight. *Metabolism of 3-methoxytyramine-1-C^{14}.* A group of 6 rats was treated with 100 mg of iproniazid/kg, and 25 μg of 3-methoxytyramine-1-C^{14}, were injected daily. Urine samples were collected for 24-hour periods during 6 days. Urine was treated as described above and radioactive 3-methoxytyramine and 3-methoxy-4-hydroxyphenylacetic acid were isolated. Labeled 3-methoxynorepinephrine could not be detected. *Shock stress of animals.* Rats were stressed in a specially constructed "activity wheel," 15 inches diameter. The wheel was so arranged to permit a shock of moderate intensity (1.5 milliamperes) to be delivered to the feet of rats. When the shock was applied (for 10 seconds at intervals of 30 seconds) the normal response of the animals was to run at high speed to minimize the intensity of the painful stimulus. The procedure was continued for 2 to 3 minutes for each rat.

TABLE I. Mobilities of Catecholamine Triacetate Derivatives and Methoxycatecholamine Diacetate Derivatives in Bush "C" Chromatography System* at 37°C.

Acetate derivate of	Distance from starting line, cm	Mobility in cm/hr
norepinephrine	7- 9	2
methoxynorepinephrine	11-13	3
epinephrine	18-20	4.75
methoxyepinephrine	20-22	5.25
3-hydroxytyramine	23-25	6
3-methoxytyramine	27-29	7

* Descending technic with Whatman No. 1 filter paper prewashed with benzene:methylalcohol (1:1) was used. Catecholamines were developed by passing a narrow strip of paper through solution of 1% $FeCl_3$ and 2% potassiumferricyanide which was then blotted and passed through a solution of 10% KOH in methylalcohol:water (1:1). Methoxycatecholamines were developed by passing a narrow strip through solution of 0.1% dichloroquinone chlorimide in alcohol, blotting and then passing it through a solution of 0.1 m borate buffer pH 10, blotting again and exposing the strip to ammonia vapors.

Results. Mobilities of acetylated catecholamines and methoxy-catecholamines in the "C" solvent system of Bush are listed in Table I. The methoxy derivative moves somewhat faster than its corresponding catecholamine.

A radiochromatogram of the acetylated urine fraction from rats which had been treated for only one day with iproniazid and hydroxytyramine-1-C^{14} shows 2 radioactive peaks with the same mobility as 3-hydroxytyramine triacetate and 3-methoxytyramine diacetate (Fig. 1). However, after the same treatment was prolonged for 3 days, an additional radioactive peak was detected (Fig. 2) which had the same mobility as 3-methoxynorepinephrine diacetate.

When rats were stressed after one day of

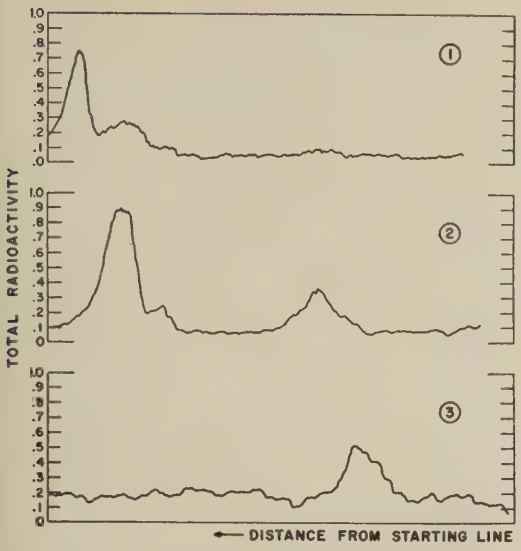

FIG. 1. Paper chromatography separation of acetylated amine fraction in urine of rats treated one day with 100 mg/kg of iproniazid and 100 μg 3-hydroxytyramine-1-C^{14}.

FIG. 2. Paper chromatography separation of acetylated amine fraction in urine of rats treated 3 days with 100 mg/kg of iproniazid and 100 μg 3-hydroxytyramine-1-C^{14}.

FIG. 3. Paper chromatography separation of acetylated amine fraction in urine of stressed rats treated for first 3 days with 100 mg/kg of iproniazid and 100 μg 3-hydroxytyramine-1-C^{14}, and for the last 2 days with iproniazid only.

A 24 hr urine specimen was collected at end of treatment period.

treatment with iproniazid and 3-hydroxytyramine-1-C^{14}, the amine fraction yielded the same 2 radioactive peaks as in the unstressed rats. However, a relatively higher excretion of labeled 3-methoxytyramine was found in stressed rats. When rats were stressed 2 days after last injection of 3-hydroxytyramine-1-C^{14}, all radioactivity of the amine fraction excreted in the next 24 hours was associated with 3-methoxynorepinephrine diacetate (Fig. 3). Table II shows radioactivity of 3-methoxynorepinephrine under various experimental conditions. It is evident that 3-methoxynorepinephrine is still excreted 8 days after last injection of 3-hydroxytyramine.

The radiochemical purity of 3-methoxynorepinephrine was established by rechromatography and deacetylation with hog kidney acylase to *N*-acetyl 3-methoxynorepinephrine. After chromatography in the "C" system of Bush for 24 hours, radioactivity was associated with *N*-acetyl 3-methoxynorepinephrine. The same sample was chromatographed in amyl alcohol water overnight, and then in chloroform formamide solvent, and radioactivity was still found to be associated with *N*-acetyl methoxynorepinephrine. The lower recovery of radioactivity after deacetylation and rechromatography (Table II) partially reflects a loss from the procedure, but is also a result of increased purity, since rechromatography of the *N*-acetyl compounds results in a better separation of norepinephrine from 3-methoxynorepinephrine.

Another radioactive sample of acetylated 3-methoxynorepinephrine diacetate was diluted with 30 mg of carrier of nonradioactive 3-methoxynorepinephrine diacetate (m.p. 123°-125°) and after 2 recrystallizations from ethyl acetate constant specific activity was obtained.

Discussion. Formation of 3-methoxynorepinephrine *in vivo* may proceed by 2 possible pathways, (a) hydroxylation of 3-hydroxytyramine to norepinephrine followed by *O*-methylation to 3-methoxynorepinephrine, or (b) *O*-methylation followed by hydroxylation. Since 3-methoxy-tyramine-1-C^{14} could not be converted into 3-methoxynorepinephrine, it must be concluded that formation of 3-methoxynorepinephrine proceeds through the first pathway, and 3-methoxynorepinephrine

TABLE II. Radioactivity of 3-methoxynorepinephrine Derivatives Isolated from 24 Hour Rat Urine by Paper Chromatography.*

Days after completion of treatment‡	Radioactivity, cpm†		
	3-methoxynorepinephrine diacetate		N-acetyl 3-methoxynorepinephrine stressed
	Unstressed	Stressed	
0	1950§	4600§	2450
2	1200	3100	1900
4	1050	2600	1100
6	850	2000	750
8	620	1300	550

* Rats were treated with 100 mg/kg of iproniazid and 100 μg of 3-hydroxytyramine-1-C^{14} daily for 3 days. Iproniazid alone was then continued until urine was collected.

† Recovery as 3-methoxynorepinephrine averaged 70-90% of the total radioactivity excreted in the urine with the exception of (§) whose values were .37 and .85% respectively.

‡ On day designated, electric shock was given to stressed rats and a 24 hr urine sample was collected from stressed and unstressed rats.

is the last step in biosynthesis of norepinephrine. The finding that 3-hydroxytyramine is a precursor of norepinephrine is in agreement with many studies *in vitro*. However, these findings are the first evidence of synthesis of norepinephrine from 3-hydroxytyramine *in vivo*. It is known that certain stresses increase excretion of norepinephrine(8), and in our stress experiments a higher excretion of 3-methoxynorepinephrine was also observed.

Of special interest may be the discrepancy in the biological half-life of 3-hydroxytyramine and norepinephrine. It was found that labeled 3-hydroxytyramine was no longer excreted 48 hours after last injection of 3-hydroxytyramine-1-C^{14}. However, radioactive 3-methoxynorepinephrine was still being excreted 8 days after last injection of 3-hydroxytyramine. Although a more quantitative study is necessary to permit exact calculation of turnover rates of these 2 catecholamines, it is evident that their half-life is different.

The fact that norepinephrine-C^{14} is stored for at least 8 days, while 3-hydroxytyramine-1-C^{14} appears to be completely replaced by synthesis of endogenous hydroxytyramine in 48 hours, suggests that the turnover rate of dihydroxyphenylalanine to hydroxytyramine is faster than the turnover of 3-hydroxytyramine to norepinephrine. This may mean that conversion of hydroxytyramine to norepinephrine is the rate-limiting step in the synthesis.

Summary. 1. Formation *in vivo* of norepinephrine from 3-hydroxytyramine-1-C^{14}, can be demonstrated indirectly by isolating the end product of biosynthesis, namely, 3-methoxynorepinephrine. 2. Labeled 3-methoxynorepinephrine was isolated as a diacetate derivative from urine of unstressed and stressed rats treated with iproniazid and 3-hydroxytyramine-1-C^{14}. Radiochemical purity of the diacetate and the *N*-acetyl derivative was established by paper chromatography, and by recrystallization of the diacetate derivative to constant specific activity. 3. Radioactivity of 3-methoxynorepinephrine isolated from urine of rats was higher in stressed rats. 4. Labeled 3-methoxynorepinephrine continued to be excreted for 8 days after treatment with 3-hydroxytyramine-1-C^{14} in contrast to radioactive 3-hydroxytyramine and 3-methoxytyramine which disappeared from urine after 48 hours.

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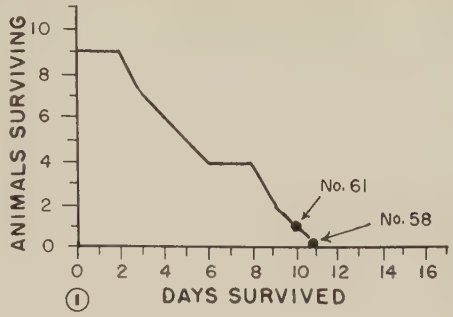
Rabies Virus Isolated from Brown Fat of Naturally Infected Bats. (25438)

J. FREDERICK BELL AND G. JOHN MOORE (Introduced by C. L. Larson)
Dept. of H.E.W., P.H.S., N.I.H., Nat. Inst. of Allergy and Infectious Diseases, Rocky Mt. Lab., Hamilton, Mont.

The physiological nature of brown fat is obscure, but is now receiving increased attention primarily because of numerous isolations of several kinds of viruses from brown fat of various animals(1) and an inference that it may play a special role in host-virus relationship(2). Sulkin *et al.*(1) adduced evidence from bats experimentally infected with rabies virus that this virus may be present in brown fat when absent from other organs, and that it may propagate in this tissue. We here present evidence of the presence of rabies

virus in brown fat of naturally infected bats: that is in pooled brown fat and pooled brain of 2 *Myotis lucifugus* and in brown fat, brain, and salivary gland of one *Eptesicus fuscus*.

Materials and methods. Swiss white mice, 21 days old (weanling), of Rocky Mountain Laboratory strain, were used routinely for isolation. Two-day-old mice were also injected in tests repeated when presence of virus in low titer was suspected. Tissues tested were brain, salivary glands, and brown fat. The last 2 tissues are near each other anatomically but grossly discrete and distinct in the 2 genera under consideration and the chance of contamination of one by the other at necropsy is remote. After bats were dead, tissues were ground by mortar and pestle in 10% albumin-saline diluent at pH 7.5 to make suspensions estimated to be 10%, and 0.03 ml were injected into mice by intracerebral route. Excess suspension was preserved in mechanical deep-freezer. Bats of the species *Myotis lucifugus* and *Eptesicus fuscus* were obtained in routine collections from attics of homes in western Montana. They were held overnight in battery jars with water available. The day following collection, they were hand-fed canned dog food* to which about 10% of an homogenate of meal worms, fresh guinea pig liver and vitamins† was added. Immediately following feeding they were banded and released into indoor flight cages (4′4″ x 5′ x 16″). No special provision was made for temperature or humidity control. The usual course of events when *E. fuscus* and *M. lucifugus* are treated thus is that an initial high mortality for several days is followed by slow attrition of survivors over several months. The group of *Eptesicus* from which the infected animals were obtained was not exceptional in this regard but the small group of *Myotis* succumbed rapidly. Signs of rabies were not observed in the infected bats and it cannot be stated that their deaths were caused by rabies. The status of infection in the animals can be inferred only to a limited extent by comparing the time of their deaths with that of other bats in the cages (Figs. 1 and 2).



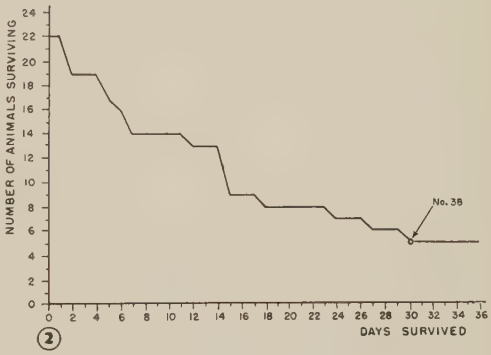


FIG. 1. Mortality in group of *M. lucifugus*.
FIG. 2. Mortality in group of *E. fuscus*.

Results. *Myotis* bats No. 61 and 58 died on 10th and 11th days of captivity, respectively, and were the last of the group to die. Carcasses were kept at 4°C until one day after death of No. 58, when they were necropsied and like tissues were pooled and injected. Rabies virus was isolated from pooled brain and from pooled brown fat after incubation periods of 16 and 18 days respectively. Five of 6 mice became infected from brain tissue and 1 of 6 from brown fat. Typical Negri bodies were found in mouse brains. Pooled salivary glands failed to infect mice on primary inoculation, and the preserved glands also failed to infect suckling mice in a subsequent test.

One hemisphere of each of the *Myotis* brains was fixed in Zenker's fluid and stained with Williams' stain. One atypical Negri body, *i.e.*, without internal structure, was found in the brain of No. 58. No other lesions could be detected.‡

Eptesicus (No. 38) died on 30th day of

*Pard.

†Abdec brand.

‡ Pathology by Dr. L. L. Ashburn, Nat. Inst. Health.

captivity and was necropsied the same day. One of 6 mice injected with brown fat suspension and all mice injected with brain or salivary gland suspensions died of rabies. Incubation periods were 22, 9, and 11 days, respectively. Titers of virus in *Eptesicus* brain and salivary glands were log 1.2 and 3.4/0.03 ml respectively. Negri bodies were not found in mouse brains. Sections of *Eptesicus* brain fixed in formalin were stained with azure-eosin and with hematoxylin and eosin. No pathognomonic changes could be discerned.

The long incubation periods of isolates from brown fat of *Myotis* and *Eptesicus* were shortened to 10 days in the second mouse passage.

Discussion. Our demonstration of rabies virus in brown fat of naturally infected insectivorous bats agrees with the findings of Sulkin *et al.*(1) who worked with experimentally infected bats. Presence of virus in brown fat when absent from salivary glands was also noted by us and by Sulkin, but in our observations the difference may not have been significant, inasmuch as only a minimal amount of virus was found in the brown fat of *Myotis*. It is quite possible that deaths of these animals were unrelated to infection inasmuch as other bats maintained identically also died but without infection. Dubiety regarding infection as cause of death may be indicated also because of absence of encephalitic lesions in the brain.

Rabies virus has been isolated by us from 12 bats of 7 species collected in western Montana. In only 2 of those instances have we attempted isolation from brown fat and both attempts were successful. We also tested tissues of 106 *Myotis, Eptesicus,* and *Corynorrhinus* but have not found virus in brown fat when absent from both brain and salivary glands.

A discussion of the role of brown fat in pathogenesis of rabies may be found in the paper by Sulkin *et al.*(1).

Summary. Rabies virus has been isolated from brown fat of naturally infected *Eptesicus* and *Myotis* bats. In each case virus was present in very low infective concentration. Rabies virus was also isolated from brains of the same bats and from salivary glands of the *Eptesicus*.

Addendum. Rabies virus has since been isolated from brown fat in 5 more instances including 3 from species not mentioned above. Two isolates were from *Lasionycteris noctivagans* and one from *M. evotis.*

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Susceptibility Rhythm to *E. coli* Endotoxin and Bioassay.* (25439)

FRANZ HALBERG, EUGENE A. JOHNSON, BYRON W. BROWN AND JOHN J. BITTNER
Divisions of Cancer Biology and Biostatistics, University of Minnesota, and Cambridge State School and Hospital, Cambridge, Minn.

In toxicity testing, variations of "response" along a 24-hour time scale are usually not evaluated. Most reviews or books on the subject actually omit discussions of physiologic rhythms in relation to bioassays in general, since available references are sporadic and questionable. The contribution of physiologic rhythms to variability of assays thus awaits rigorous evaluation, the outcome of which will depend largely upon control of sources of variation other than rhythms, such as genetics, past history, physical environment as well as sampling effects. Control of such factors was attempted in this study on the significance and reproducibility of a possible susceptibility rhythm to endotoxin. Can the latter rhythm be unmasked from interference

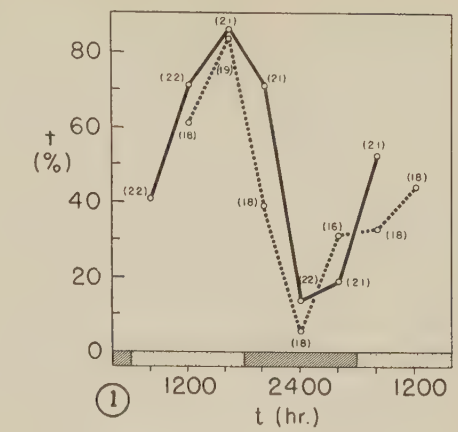
* Supported by Elsa U. Pardee Fn., Am. Cancer Soc., U.S.P.H.S., and Dept. of Public Welfare, State of Minnesota.

by other factors so that periodic changes in the body's response to a poison stand out in clear view of all? This aim was approximated by light-synchronized periodicity analysis: a dramatic susceptibility rhythm was found. In view of its reproducibility and significance under the stated conditions, its bearing upon bioassay also was experimentally explored.

Materials and methods. C (Bagg albino) mice, brother-to-sister mated for over 10 years in our laboratory, were studied. For at least 7 days prior to each experiment, the mice were singly caged at 24°C, in light daily from 06 to 18, with food and water continuously available. In each of 2 experiments, 7 groups of mature animals matched by age, sex, and weight were injected intraperitoneally at 4-h intervals with 100 μg/.2 cc/20 g body weight of Difco's *E. coli* lipopolysaccharide.† A third experiment consisted of 2 LD_{50} assays done 12 hours apart in mice that were again comparable in terms of genetic background, sex, age, past history, and experimental conditions.

Results. At 1 week post injection, as well as at earlier time-points, differences in mortality among some groups of mice injected at different times were large and significant below 1%; equally important, with the dose and stock used, these differences were reproducible from first to second experiment (Fig. 1), probably because of standardization of experimental conditions.

In the third experiment, a large difference in "potency" was seen in 2 assays made 12 hours apart with samples from the identical batch (Fig. 2); the computed "relative potency" of the same material tested at 12 and 24 was 3.22. This inter-assay difference is clearly a function of stage of susceptibility rhythm in which the injections were made. A comparison of responses at the 2 dose levels common to both assays demonstrated the significance of the difference ($P<.001$). Under our conditions, the stage of rhythm selected for testing was a much more significant source of variation than several other factors tested, such as position of cage on rack, or the type



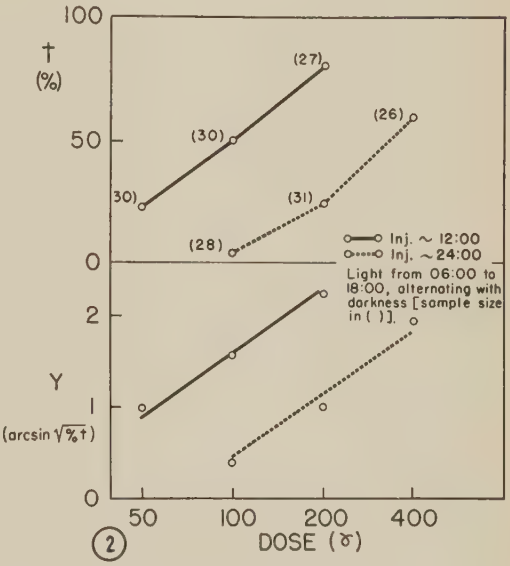


FIG. 1. Susceptibility rhythm to endotoxin and its reproducibility in separate experiments. Ordinate: % death from Difco's *E. coli* lipopolysaccharide (100 μg/20 g, i.p.) in separate groups of *standardized* mature C mice inj. at 4-hr intervals. Abscissa: times of inj. in 2 exp. begun at different times during daily light period. No. of mice/group in parentheses. Evaluation at 1 wk post inj.

FIG. 2. Different "potency" of samples from same endotoxin batch tested at different times, shown by mortality of C mice inj. in different phases of their daily susceptibility rhythm. Evaluation at 18 hr post inj.

(not the schedule!) of illumination tested (results not shown; $P>.10$).

Discussion. Standardization for light-dark synchronized periodicity analysis is desirable for bioassay as well as for other experimental work(1): Variability will be reduced by testing synchronized populations in defined phases

† The authors are greatly indebted to Dr. A. L. Lane of Difco Labs., Detroit, Mich. for ample supplies of endotoxin.

of 24-hour rhythm. In the absence of such standardization intra-assay variability may be augmented by dissociation of susceptibility rhythms among individuals and inter-assay variability by testing in different stages of rhythm.

Control of clock-hour of testing does not necessarily suffice for reducing the contributions of rhythms to variability of bioassays; rhythms might be free-running from the local clock with periods slightly though consistently different from 24 hours(1,2). Standardization for light-synchronized periodicity analysis overcomes this difficulty: "physiologic time" in terms of stage of susceptibility rhythm can then be related to a given clock hour. Subsequent work shows further that timing of peak (or trough) in susceptibility to endotoxin can be shifted from the usual clock hour by appropriate manipulation of the lighting regimen and by observing several additional simple precautions such as avoidance of disturbances in the mouse room, *e.g.*, from presence of experimenters(1). Work carried out in reproducibly defined stages of a given physiologic rhythm is particularly critical when it allows for detection (in one stage of rhythm) of effects which might not otherwise be found (in tests done in another stage of rhythm)(3). Apart from methodologic considerations, susceptibility rhythms show how critically the stage of *periodic* changes in physiologic state can determine an organism's ability to withstand damage.

Summary. Light-synchronized periodicity analysis reveals a susceptibility rhythm in C mice to *E. coli* endotoxin. Susceptibility varies predictably and significantly along the 24-hour time scale. A dose of endotoxin which is compatible with survival of most animals when given during middle of daily dark period is highly lethal when it is given 8-12 hours earlier or later. LD_{50} also was determined for 2 samples from identical batch of endotoxin tested 12 h apart on separate groups of comparable C mice kept under conditions standardized for periodicity analysis: Differences in potency significant at 1% were seen at 2 dose levels common to both assays, the computed "potency ratio" being 3.22.

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Inhibitory Influence of Ethanol on Serotonin Metabolism. (25440)

GEORGE ROSENFELD* (Introduced by A. P. Krueger)

Dept. of Physiology and Naval Medical Research Unit No. 1, University of California, Berkeley

During a study of acute experimental intoxication using normal human subjects, it was observed that a pronounced decrease in urinary excretion of 5-hydroxyindoleacetic acid (5-HIAA) occurred during 5-hour collection period following ingestion of about 100 g of ethanol. Inasmuch as 5-HIAA is the major metabolic end-product of serotonin, (5-hydroxytryptamine) metabolism in man(1,2), this finding conjured up the possibility that during metabolism of alcohol the oxidative conversion of endogenous serotonin (and, perhaps, of other monoamines) is temporarily inhibited. Further circumstantial evidence in support of this hypothesis was adduced from preliminary pharmacological studies using mice, which demonstrated that doses of serotonin and other primary aromatic amines, *viz.*, tryptamine, dopamine, and tyramine, which *per se* were innocuous and provoked little somatic, autonomic, or behavioral effects, exerted a striking potentiation of the narcotic action and acute toxicity of alcohol. Prompted by the possibility that the neuropharmacologic

* Opinions or assertions contained herein are private ones of author and are not to be construed as official or reflecting views of Navy Department or naval service at large.

and toxicologic effects of alcohol may be related, at least in larger doses, to its influence on endogenous monoamine metabolism, the present studies were undertaken to ascertain whether a sublethal, hypnotic dose does, in fact, interfere with the metabolism of serotonin. The experimental design involved parenteral administration of serotonin to mice pretreated with alcohol, and subsequent analysis at various intervals of the whole animal, including urine and feces. The latter procedure was adopted in preference to analyses of excreta alone because even moderate doses of alcohol can induce acidosis. Accordingly, a diminution observed only in the urinary output of metabolites like 5-HIAA could be attributable merely to a decreased renal excretion rather than a suppression of their formation.† Regarding analytical methods employed, determinations were conducted of serotonin and total 5-hydroxyindole compounds as well as of 5-HIAA because, in mammalian species other than man, 5-HIAA production accounts for only a portion of the serotonin destroyed and alternate metabolic routes must be considered. In view of probable formation of unidentified conjugation products, disappearance of serotonin and formation of 5-HIAA were followed directly. Moreover, since both of these are 5-hydroxyindole compounds, it was believed that measurement of total 5-hydroxyindoles would provide added information *vis-a-vis* the influence of alcohol on alternate metabolic routes involving conjugation.

Materials and methods. Adult male albino mice, NAMRU No. 1 strain(3), weighing 25 to 30 g, were employed. The experimental ones were pretreated intraperitoneally with 4.5 g/kg of a 25% (w/v) ethanol solution in 0.9% NaCl while control animals were given an equivalent volume of 0.9% NaCl, *i.e.*, 18 ml/kg. Preliminary studies revealed that this dose of alcohol resulted in no deaths among 40 treated mice, and that the mean period of hypnosis, defined as time from loss to return of the righting reflex, was 74 minutes. Under present conditions, doses up to 5.5 g/kg produced no deaths, and LD_{50} was between 6.5 and 7.0 g/kg. Thirty minutes after alcohol or saline control injection, mice were administered 160 mg/kg of serotonin intraperitoneally, and placed directly into beakers. The serotonin solution, freshly prepared by dissolving the creatinine sulfate complex (obtained from California Corp. for Biochemical Research) in 0.9% NaCl, was carefully brought to pH 7.0 with NaOH before use. At specified times following serotonin injection, groups of 4 mice together with their excreta, thoroughly washed out of the beakers, were placed in a Waring blendor and homogenized for 4 minutes in a total volume of 0.1 N HCl 4-fold their combined weight. Thus, each 5-ml aliquot represented 1 g of body weight and theoretically would contain 160 μg serotonin if obtained from mice homogenized immediately following injection. After straining through several layers of gauze, 25-ml portions of these homogenates were mixed with 5 ml of 10% $ZnSO_4 \cdot 7H_2O$ and 2.5 ml of 1N NaOH to effect a neutral protein precipitation. Clear supernates were obtained upon centrifugation for 10 minutes at high speed. Methods employed to determine serotonin, 5-HIAA, and total 5-hydroxyindoles in duplicate aliquots of these supernates are based on the Gerngross reaction with 1-nitroso-2-naphthol. They were modifications of those reported by Udenfriend and coworkers(1,4), who found that this reagent, in dilute HCl or H_2SO_4 containing traces of nitrite, will react almost exclusively with 5-hydroxyindoles to form a violet chromophore. Of the many phenolic and indole compounds tested, p-hydroxyacetanilide and mephenesin are the only known ones not 5-hydroxyindoles, which yield a violet color. Whereas measurement of serotonin and 5-HIAA, both of which are 5-hydroxyindoles, required preliminary separation and isolation from the supernates by means of successive extractions and partitions, colorimetric determination of total 5-hydroxyindoles was carried out directly on the filtered supernates. A blank and inclusive series of standards were concurrently run with each set

† In human intoxication study already cited, urinary excretion of uric acid was also markedly decreased. This finding is possibly related to alcohol-induced acidosis, for it is well known that in conditions of acidosis, renal excretion of uric acid is diminished.

TABLE I. Influence of Ethanol Pretreatment on Amounts of Serotonin, 5-HIAA, and Total 5-Hydroxyindoles Found in Whole Body and Excreta of Mice Administered Serotonin.* (2 exp. with 4 mice for each period).†

Time after serotonin inj., min.	μg/g found						μg/g calculated	
	Serotonin		5-HIAA		Total 5-OH indoles		Conjug. & other products§	
	Control	Alcohol	Control	Alcohol	Control	Alcohol	Control	Alcohol
0	159 162		0 0		161 160		0	
30	53 (99)‡ (44.5) 113 70 119		33 (27)‡ (9) 10 21 8		85 (89.5)‡ (126) 124 94 128		72	35.5
60	38 (119.5) (75.5) 79 44 91		36 (32) (18) 22 28 14		75 (77.5) (106.5) 101 80 112		87.5	57.5
90	25 (134.5) (110) 44 27 57		35 (32) (27.5) 29 29 26		70 (72.5) (90) 83 75 97		102.5	82.5
120	13 (145.5) (129) 31 17 32		30 (28) (30) 32 26 28		61 (64.5) (79) 76 68 82		117.5	99.0
150	11 (147) (135.5) 23 14 27		28 (26.5) (28) 30 25 26		56 (61) (73) 69 66 77		120.5	107.5

* Exp. mice were pretreated with 4.5 g/kg of a 25% ethanol solution in 0.9% NaCl; control mice were given 18 ml/kg of 0.9% NaCl. Thirty min. later, all were inj. I.P. with 160 mg/kg serotonin.

† Each value represents mean of duplicate dets. conducted on pooled carcasses and excreta of 4 mice, except for those in parentheses and the last 2 columns, which represent means of 8 animals.

‡ Values in parentheses, from left to right, represent: serotonin destroyed, calculated by subtracting mean amount recovered from mean zero-time value; mean 5-HIAA found; mean total 5-OH indoles found.

§ Metabolites of serotonin other than 5-HIAA, calculated by subtracting mean amount of 5-HIAA found from mean amount of serotonin destroyed.

of determinations and carried through all extraction and color development procedures.

Results. Table I embodies analytical findings of 2 experiments on the influence of ethanol on metabolism of parenterally administered serotonin. In mice pretreated with an hypnotic, sublethal dose of alcohol, rate of destruction of exogenous serotonin was appreciably decreased. Concordant evidence for the inhibitory effect of alcohol on serotonin metabolism is provided by the following data: (1) recovery of larger amounts of unmetabolized serotonin at successive intervals following injection; (2) detection of smaller amounts of 5-HIAA, particularly in early stages of detoxification‡; (3) finding of greater quantities of total 5-hydroxyindole compounds despite the smaller amounts of contributory 5-HIAA; (4) formation of less "conjugated and other products" *i.e.*, metabolites of serotonin, other than 5-HIAA, calculated by subtracting mean amount of 5-HIAA found from mean amount of serotonin destroyed. The interrelationships of these findings are portrayed in Fig. 1.

In connection with formation of metabolites other than 5-HIAA, it is noteworthy that the amounts of 5-HIAA recovered do not account for the total quantities of serotonin destroyed. For example, after 30 minutes when 99 and 44.5 μg/g of serotonin had been metabolized by control and alcohol-treated mice, resp., only about 27 and 9 μg/g, resp., were accounted for as formed 5-HIAA. Obviously, a substantial portion of administered serotonin had been converted *via* another route(s), a probable one being conjugation of its 5-hydroxyl group. This is confirmed by total 5-hydroxyindole data which also exhibit successive declines. Theoretically, total 5-OH indole level would remain constant (around 160 μg/g) if serotonin were converted only to 5-HIAA since both are 5-hydroxyindoles. In view of its progressive fall, it may be concluded that serotonin disappears not only as a consequence of oxidation of its aliphatic side chain but also *via* conjugation of its aromatic 5-OH group, *e.g.*, glucuronide formation. Only recently, Weissbach and cowork-

‡ As indicated by data of Table I, 5-HIAA itself probably undergoes further alteration, *e.g.*, oxidation, conjugation, etc. These conversions, particularly by conjugation, may also be inhibited by alcohol.

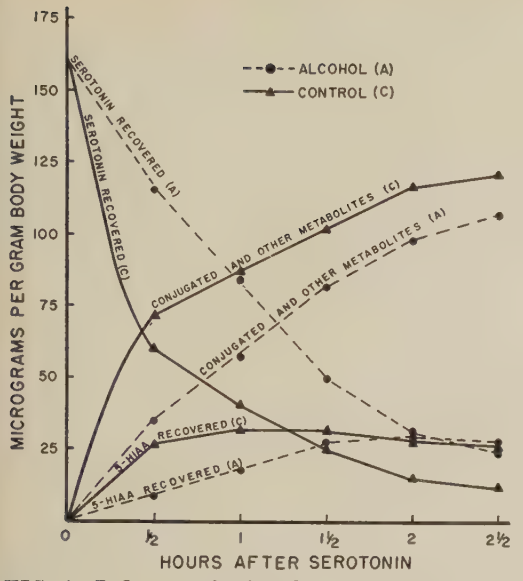

FIG. 1. Influence of ethanol pretreatment on metabolism of serotonin parenterally administered to mice. Thirty min. after I.P. inj. of 4.5 g/kg ethanol, mice were given 160 mg/kg serotonin I.P. At various times after the serotonin dose, the whole animal and its excreta were homogenized and assayed for serotonin, 5-HIAA, and total 5-OH indoles. The "conjugated and other metabolites" represent products derived from serotonin other than 5-HIAA. Each point represents mean value for 8 mice.

ers(5) reported that serotonin-o-glucuronide was, in fact, a normal, major metabolite which accounted for at least 25% of a dose of C^{14}-labeled serotonin administered to mice. Thus, data of Table I reveal that, in addition to inhibiting oxidation of serotonin to 5-HIAA, narcotic doses of alcohol depress its biotransformation *via* glucuronide conjugation.

Perhaps, one more observation should be made from data in Table I, concerning total 5-hydroxyindole compounds measured. If the total 5-OH indoles comprised only serotonin and 5-HIAA, its value should never be greater than their summation. However, one finds that it is. In fact, as detoxification of serotonin progresses, there is an increasing disparity between total 5-OH indoles found and the sum of serotonin and 5-HIAA recovered. This strongly suggests that, in addition to 5-HIAA and serotonin-o-glucuronide, there are metabolites of serotonin which possess an unconjugated 5-OH aromatic group and an oxidized (other than acetic), and/or oxidized (including acetic) plus conjugated, and/or conjugated (or methylated?) aliphatic side chain. It is possible that this group constitutes an even greater proportion of unidentified end-products than is indicated by the 5-OH indole data inasmuch as conjugation, methylation, etc. of the side chain probably interferes with intensity of the 5-OH indole color reaction.

Discussion. The present findings revealed that a sublethal, hypnotic dose of alcohol in the mouse inhibited metabolism of a parenteral dose of serotonin, and depressed its biotransformation *via* oxidation and conjugation. The oxidative conversion of serotonin involves its deamination by monoamine oxidase to an intermediate aldehyde and subsequent oxidation of the latter by means of aldehyde dehydrogenase and DPN to 5-HIAA(6). Moreover, both stages of ethanol oxidation require DPN as a coenzyme, and the second stage utilizes the same enzymatic machinery involved in oxidative conversion of the aldehyde formed from serotonin. The preceding enzymatic considerations suggest that during the initial period, when rate of alcohol metabolism was maximum, its oxidation competitively inhibited the oxidative metabolism of serotonin, and perhaps, in particular, oxidation of the aldehyde derived from it. Under such conditions, one might expect that more serotonin would be metabolized *via* an available, alternate route, *e.g.*, conjugation. However, concurrent depression of this metabolic route occurred. Perhaps, competitive inhibition also underlies the mechanism by which ethanol delays conjugation of serotonin in view of the fact that DPN is involved in glucuronic acid formation, and that small amounts of ethanol are metabolized *via* glucuronic acid conjugation(7).

Although inhibition of only serotonin metabolism was demonstrated by the present chemical data, it is reasonable to assume that detoxification of other primary aromatic monoamines would be similarly affected inasmuch as they are inactivated *via* the same metabolic route(s). Endogenous amines like dopamine, tryptamine, tyramine (and to a lesser extent, norepinephrine and epinephrine) are all good substrates for the monoamine oxidase of liver, brain, intestine, kidney and other

tissues, and are oxidatively deaminated by it to corresponding aldehydes. Other circumstantial evidence in support of this hypothesis is provided by the pharmacological studies already cited, which showed that large amounts of serotonin, tryptamine, dopamine and tyramine could be injected intraperitoneally in the mouse without provoking hypnosis or death. However, if animals were pretreated with the same hypnotic dose of alcohol as presently employed, even a moderate dose of these amines induced a substantial mortality as well as a decisive prolongation of sleeping time. In fact, extremely small doses, which *per se* provoked little somatic, autonomic, or behavioral effects, induced a marked potentiation of the narcotic action of alcohol despite the fact that neither brain alcohol content nor overall body rate of alcohol destruction were modified.

Inasmuch as the amines employed in these experiments as well as in the present ones were administered intraperitoneally, the liver was probably the main site of their metabolism, as it is of alcohol, in general. Together then, chemical and pharmacological findings strongly suggest that large doses of alcohol may impair, at least temporarily, the functional capacity of liver to detoxify serotonin and other primary aromatic monoamines. Thus, they differ from the majority of studies reported which revealed little or no demonstrable functional impairment in intoxicated men subjected to a battery of liver function tests. It is the usual custom, at present, to regard pathological alterations in the liver and other organs of chronic alcoholics as malnutritional sequelae or complications rather than ascribe them to a direct effect of alcohol. In view of the accepted role of liver as an inactivator of endogenous amines and perhaps, some exogenous ones formed in the intestinal lumen by bacterial activity, it is tempting to implicate a chronic alcohol-induced suppression of aromatic monoamine metabolism in the etiology of degenerative changes in this organ after years of excessive and habitual ingestion of alcohol.

Theoretically, the monoamine metabolism of the brain§ should also be influenced by levels of alcohol which affect liver monoamine metabolism inasmuch as it does not possess multiple routes of amine inactivation and depends mainly on oxidative conversion *via* its monoamine oxidase content. Within the last few years a number of aromatic monoamines, *viz.*, serotonin, norepinephrine, dopamine, and tryptamine, have been shown to occur naturally and selectively in the brain. Moreover, there is a growing body of evidence that some of these endogenous monoamines may act as neurohumoral agents in the regulation of central transmission in certain areas. While the possibility still cannot be precluded that narcotic properties of alcohol are derived from a primary interference with some stage of neuronal oxidative and/or high-energy bond metabolism, it appears reasonable to postulate that, at least in higher concentrations, there is a casual relationship between the neuropharmacologic action of alcohol and its influence on monoamine metabolism.

Summary. The influence of a sublethal, hypnotic dose of ethanol on metabolism of serotonin was investigated in the mouse. Thirty minutes after intraperitoneal injection of 4.5 g/kg of ethanol, mice were administered 160 mg/kg of serotonin intraperitoneally. At various intervals after the serotonin dose, the whole animal and its excreta were homogenized and analyzed for serotonin, 5-HIAA, and total 5-hydroxyindole compounds. The results clearly demonstrated that alcohol exerted an appreciable inhibitory effect on metabolism of serotonin and depressed its biotransformation *via* oxidation and conjugation. The enzymatic, toxicologic, and neuropharmacologic implications are discussed.

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§ Of all body tissues, the brain of a mouse administered iproniazid, a potent monoamine oxidase inhibitor, exhibits the most striking effects either in endogenous serotonin metabolism or after 5-hydroxytrytophan administration.

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Thyroid Hormone and Mammary Gland Growth in the Rat.* (25441)

RICHARD C. MOON† AND CHARLES W. TURNER
(With technical assistance of Mary E. Powell)
Dept. of Dairy Husbandry, University of Missouri, Columbia

The beneficial effect of thyroid hormone and thyroid active substances upon lactation is well established(1), however, little is known concerning the role of this hormone in influencing growth of mammary gland. Lactation studies have shown that if hormones influencing lactation were administered in optimal amounts, increased milk yields occurred with marked reduction in variability of response among members of the population(2,3). Similarly, any hormone, if present in suboptimal amounts, may also limit mammary gland growth or, at least, contribute to variability in growth which occurs during normal pregnancy or to that of glands developed experimentally with optimal levels of estrogen and progesterone(4). In the present study we determined the effect of mild hyperthyroidism upon experimental development of rat mammary gland using desoxyribosenucleic acid (DNA) as an index of growth.

Materials and methods. Groups of ovariectomized rats of the Sprague-Dawley-Rolfsmeyer strain with an initial weight of 225-275 g were used. Body weight was recorded daily. Fifteen ovariectomized rats killed 34 days post-castration served as controls. Remaining ovariectomized rats received daily subc. injections for 19 days commencing 14 day post-castration as follows: 1) 18 received either 1 or 2 μg estradiol benzoate (EB); 2) 29 were injected with 1 μg EB and 3 or 6 mg progesterone (P); 3) 13 received 2 μg EB and 6 mg P; 4) 31 were treated with 1 μg EB, 3 mg P and 3 or 6 μg l-thyroxine; and 5) 49 received 2 μg EB, 6 mg P and 3 or 6 μg thyroxine. Rats were killed one day after last injection, skinned rapidly and abdominal-inguinal glands removed and immediately frozen for 4 days. Tissues were then thawed and fat extracted in boiling 95% ethanol for 6 hours followed by ether extraction for another 6 hours. DNA was extracted from 25 mg aliquots of finely ground, dry, fat-free tissue (DFFT) by the method of Schneider(5) with exclusion of the fat extraction and cold trichloroacetic acid (TCA) extraction steps. DNA was determined by the method of Webb and Levy(6). The product of the quantity DNA/mg DFFT and total DFFT, expressed as unit body weight, was estimated as the total DNA/posterior 6 glands. Visual observations were made for presence of lobule-alveolar development but no attempt was made to visually quantitate extent of growth.

Results. Total DNA of glands of ovariectomized rats receiving 2 μg EB for 19 days did not differ significantly from those of animals receiving 1 μg EB (Table I). Injections of 1 μg EB and 3 mg P increased mammary gland DNA above that of rats receiving EB alone, but upon increasing EB and P levels to 2 μg and 6 mg, respectively, no significant increase in growth occurred. Addition of 3 or

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† Postdoctoral Fellow of N.I.H. This investigation supported in part by grants from P.H.S. and Am. Cancer Soc. Present address: Dept. of Physiology, University of Tennessee, Memphis.

TABLE I. Mammary Gland DNA of Adult Rat Ovariectomized for 14 Days Followed by Estradiol Benzoate (EB), Progesterone (P) and Thyroxine (T_4) for 19 Days.

Treatment (amt/day)	No. of rats	Mean: Body wt (g) Initial	Body wt (g) Terminal	DFFT* (mg)	DNA/mg DFFT (μg)	Total DNA, posterior 6 glands (μg/100 g)
Ovariectomized controls†	15	263.0	273.7	359.2	23.20 ± .54‡	3.05 ± .14[1]
1 μg EB†	5	239.6	246.8	485.6	22.86 ± 1.27	4.48 ± .15[2]
+ 3 mg P†	19	250.0	269.0	590.1	35.65 ± 1.09	7.72 ± .25[3]
+ 6 "	10	253.6	278.4	628.1	35.50 ± 1.53	7.95 ± .34
+ 3 " + 3 μg T_4	19	268.2	270.0	558.6	39.77 ± 2.52	8.03 ± .28
+ 3 " + 6 "	12	238.5	248.1	384.7	47.05 ± .72	7.26 ± .27
2 μg EB	13	269.8	267.8	462.9	24.35 ± .94	4.11 ± .22[4]
+ 6 mg P	12	270.3	272.0	592.6	36.63 ± 1.51	8.13 ± .42[5]
+ " + 3 μg T_4	35	251.6	253.0	662.9	38.47 ± 1.18	9.87 ± .27[6]
+ " + 6 "	14	253.8	256.7	592.1	44.46 ± 3.01	9.95 ± .41[7]

* Dry, fat-free tissue.
† Data from Moon *et al.*(4)
‡ Stand. error of mean.

Students "t"	Probability
1–2	.001
2–3	"
3–6,7	"
5–6,7	.005

6 μg thyroxine to the hormone regime has no beneficial effect upon mammary gland growth at the 1 μg EB and 3 mg P level, whereas thyroxine administered concomitantly with 2 μg EB and 6 mg P results in a significant increase in mammary gland DNA. Body weight increased in all groups except those injected with 2 μg EB alone. Animals receiving 2 μg EB concomitantly with the other hormones maintained their body weight throughout the experimental period. Visual examination of mammary glands of rats treated with EB alone revealed a well developed duct system with endbuds and side branch formation. Animals receiving EB and P and/or thyroxine exhibited excellent lobule-alveolar development.

Discussion. It has been suggested that the observed variability in normal and experimental mammary gland growth may be due, in part, to endogenous secretion of sub-optimal amounts of thyroid hormone(4). If this is true, an increase in mean growth response might be expected upon administration of thyroid active substances. However, as thyroid hormones are general metabolic stimulants, it is important that the levels administered do not cause stress to the animal. In the present study, daily administration of thyroxine at levels slightly above the highest TSR (2.5 μg/100 g) previously observed in our rats(7) did not adversely affect body weight indicating that the levels employed did not produce severe hyperthyroidism. Concomitant administration of 3 or 6 μg/100 g thyroxine with 1 μg EB and 3 mg P had little beneficial effect upon mammary growth, although it had been shown these levels of ovarian steroids produce growth comparable to that of rats pregnant 18-20 days(4). Upon doubling the steroid levels, thyroxine resulted in a significant increase in total DNA which did not occur when the higher levels of EB and P were administered alone. The failure to observe enhanced growth at the low dosage of EB and P may be due to increased metabolic removal (half-life) of these steroids. It is known that removal rate of cortisol is markedly elevated in hyperthyroidism. The increased removal rate of cortisol has been attributed to an increase in metabolic activity of hepatic cells(8). Since the liver is also the principal site of estrogen (E) and P metabolism, it is likely that removal rates of these steroids are also elevated. Increased metabolic removal of E and P, which normally exhibit a very short half-life (9), would tend to reduce the effective circulating level of these steroids below that which is necessary for additional growth. The significant increase in total DNA resulting from injection of thyroxine and the higher level of ovarian steroids suggests that sufficient quantities of the hormones were available to meet the increased metabolic requirements of mam-

mary tissue. Griffith and Turner (unpublished results) have recently observed an increase in total DNA of pregnant rats injected daily with 2.5 or 3.5 μg/100 g thyroxine. It has been shown also that pregnant rats fed desiccated thyroid exhibit precocious mammary gland development(10). These data indicate that during pregnancy endogenous secretion of E and P may be sufficient to overcome the increased metabolic removal of the steroids during hyperthyroidism and as a result, mammary gland growth may be increased. Although the thyroid hormone is not essential for mammary gland growth(11), the present data suggest that variability in total mammary gland growth may be due to a deficiency in TSR or of E and P, or both. When the TSR is high, it is fully effective in stimulating the greatest mammary gland growth only when endogenous E and P secretion is adequate.

Summary. 1) The effect of mild hyperthyroidism upon mammary gland growth has been studied in adult ovariectomized rats treated with estradiol benzoate (EB) and progesterone (P) for 19 days. 2) Using total DNA as index of mammary growth, glands of animals receiving daily injections of 1 μg EB, 3 mg P and 3 or 6 μg/100 g thyroxine did not differ from that of controls. Upon doubling EB and P levels, a significant increase in total DNA was observed. 3) It is suggested that thyroid hormone may be a limiting factor in mammary gland growth when E and P secretions are adequate, but when TSR is optimal, E and P secretion may limit growth.

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Intradermal Vaccination in Healthy Adults With Type A Asian Strain Influenza Vaccine.* (25442)

MARGUERITE L. CANDLER, GERALD R. COOPER, U. PENTTI KOKKO, ROSLYN Q. ROBINSON AND MYRON J. WILLIS

Communicable Disease Center, Public Health Service, U.S.D.H.E.W., Atlanta, Ga.

The intradermal route for administration of Asian strain Type A influenza vaccine was employed widely by practicing physicians in the fall of 1957 when the demand for vaccine exceeded the supply. In view of widespread use of this method of vaccination, this study was undertaken to determine the relative efficiency of various doses of vaccine in producing measurable immune responses. In Sept. 1957 when the program was started, no clinical influenza had been reported in the study area. The vaccine used throughout was a single lot of commercially prepared monovalent Type A Asian strain influenza vaccine which contained 1000 chicken cell agglutination (CCA) units/ml. To administer different amounts of vaccine in the same volume the vaccine was diluted with pyrogen-free saline to make 4 concentrations containing 12.5, 25, 50, and 100 CCA units in 0.1 ml. Two hundred and forty-two civilian employees from

* We wish to acknowledge the cooperation of South Atlantic Corps of U. S. Engineers, who provided volunteers which made this study possible and Dr. Charles C. Rand, Supt. of Grafton State School, Grafton, N. D., who provided convalescent sera.

the Army Corps of Engineers in Atlanta between ages 20 and 60 volunteered for the study. These individuals were randomly assigned to 5 groups which received 0.1 ml vaccine intradermally in the volar surface of the forearm, (Table I). Groups I-IV received a second dose of vaccine after a 2-week interval; Group V received only one injection. In Oct. 1958, 53 weeks after initial program was begun, a booster vaccination was administered to 79 volunteers. The booster dose for each was the same size as the first dose. Venous blood was collected from all volunteers prior to administration of the first injection of vaccine (week 0), prior to second dose (week 2) and again 2 weeks later (week 4). Additional blood specimens were obtained from 75 individuals 16 weeks after first injection. During the booster phase of the program blood specimens were collected from all returning individuals just prior to vaccine injection (week 53) and again 2 weeks later (week 55).

Results. Antibody response. Antibody titers were determined by the hemagglutination inhibition test using the A2/Japan/305/57 antigen (EFME)(1,2). Antibody titrations were done by using 2-fold serum dilutions starting at 1:10. For calculation of geometric mean titers, actual titers were used for those classed as 320-1280 in Table I. Two weeks after the first injection of vaccine, there was slight but definite antibody response increasing with dose. This ranged from 15% in Group I to 47% in Group IV at 1:20 (Table I). Two weeks after the second vaccination, the percentage of individuals with antibody titers 1:20 or greater had further increased in all study groups to a range of 63 to 90% including Group V which had received only one dose. However, 16 weeks after the first dose when 75 individuals returned for blood sampling, 26 individuals (35%) with measurable antibodies at week 4 (highest titer 1:320) had lost all their detectable serum antibodies and 45 individuals (60%) had up to 16-fold decreases of titers and only 4 individuals (5%) had maintained their titers. The geometric mean titers of different groups stayed approximately on the same level between weeks 16 and 53. The booster dose in each group resulted in pronounced titer increases. (Table I).

The number of individuals decreased as the study progressed. The individuals who participated in the entire study produced titers in the initial vaccination program which did not vary significantly from the total group and therefore were considered as a representative sample.

Reactions to vaccine. No individuals with a history of sensitivity to eggs were included in this study. The individuals included did not know what dose of vaccine they were receiving. The sites of vaccine injections were observed 24 hours after first and second vaccination. An increasing erythema was noted with increased dosage, but there was no significant difference in the local reaction after the first and second injections. The mean diameter of erythematous areas was 17 mm in Group I; 20 mm in Group II; 26 mm in Group III; and 38 mm in Groups IV and V.

Careful questioning of each individual showed that greater doses caused more subjective symptoms than the smaller doses. The proportion of individuals with systemic complaints after the first injection of vaccine ranged from less than 10% in Group I to over 50% in Groups IV and V. The most frequent complaints were headaches, slight generalized muscular aches, drowsiness, dizziness, nasal congestion, chilly sensations and flushing. A few reported discomfort at site of injection. Three per cent of those in Groups I and II and 15% in groups III, IV, and V reported fever 6-24 hours after first injection. After the second injection of vaccine less generalized symptoms were reported, and still less after the booster injection. No generalized urticaria or asthma were reported.

Discussion. It is not possible to determine on the basis of this study how the antigenic effect of intradermal administration of influenza vaccine compares with subcutaneous or intramuscular injection of the same vaccine. However, one may note that the circulating antibody response to intradermal immunization in this study compares quite favorably with several other studies in which varying lots of Asian influenza vaccine were administered subcutaneously(3,4,5,6).

TABLE I. Titer Distribution Following Varying Doses of Vaccine by Intradermal Route.

Dose in CCA units	Wk after vaccination	No. of individuals								Geometric mean titers*	
		Total	<10	10	20	40	80	160	320-1280	1	2
Group I											
12.5	0	52	51	1	0	0	0	0	0	<10	<10
"	2	52	32	12	3	2	2	1	0	<10	<10
	4	39	7	7	8	12	4	1	0	21	20
	16	19	15	2	1	1	0	0	0	10	10
"	53	18	13	2	0	2	1	0	0	10	10
	55	18	0	0	5	5	3	5	0	54	54
Group II											
25.0	0	47	47	0	0	0	0	0	0	<10	<10
"	2	47	25	6	8	4	3	0	1	11	<10
	4	33	7	5	6	8	2	4	1	24	34
	16	10	9	0	0	1	0	0	0	10	10
"	53	11	7	3	0	0	1	0	0	10	10
	55	11	1	0	1	5	1	2	1	51	51
Group III											
50.0	0	50	49	1	0	0	0	0	0	<10	<10
"	2	50	26	5	11	3	4	1	0	11	11
	4	25	3	1	6	3	8	2	2	41	40
	16	14	10	2	0	2	0	0	0	10	10
"	53	15	11	1	1	1	1	0	0	10	10
	55	15	0	0	0	4	6	3	2	100	100
Group IV											
100.0	0	93	93	0	0	0	0	0	0	<10	<10
"	2	93	36	13	15	12	9	2	6	17	21
	4	20	1	1	4	6	3	2	3	51	69
	16	14	5	5	2	0	0	1	1	13	16
"	53	10	4	2	1	1	1	1	0	15	15
	55	10	0	0	0	2	0	4	4	211	211
Group V											
100.0	0	93	93	0	0	0	0	0	0	<10	<10
	2	93	36	13	15	12	9	2	6	17	16
	4	48	11	7	13	7	5	1	4	22	26
	16	18	10	2	0	3	1	2	0	13	14
"	53	25	11	4	6	2	2	0	0	11	11
	55	25	0	0	1	6	7	8	3	100	100

* 1—Includes all sera tested. 2—Includes only those individuals who returned for 53 and 55 wk blood tests.

It is probably impossible to establish any single level of circulating antibody which would represent an adequate protective titer against "natural infection." In the study of Bell and associates(7) in which vaccinated and non-vaccinated individuals were challenged with live virus, there was evidence of vaccine protection in those who had developed detectable antibody titers 1:10 or greater, following vaccine. The authors of this paper measured the circulating antibody level of 65 nonimmunized convalescents approximately 3 weeks after illness. In this group 82% of the convalescents had titers 1:20 or higher and 52% 1:40 or higher. The geometric mean titer of the convalescent group was 1:29. These antibody values compare closely with those in Groups II and III in the present study.

If the circulating antibody responses in different vaccination groups are compared, one will note that 2 doses of 50 CCA units at 2 week intervals gave better antibody response than a single dose of 100 CCA units and even 2 doses of 12.5 CCA units of vaccine resulted in a response comparable to a single dose containing 4 times as much total antigen.

Attempts were made to determine the protective value of the vaccination during the epidemic which followed in Atlanta several weeks after immunizations were completed. Although follow-up studies could not be performed on the total number of cases scattered throughout the metropolitan area, some infor-

mation was collected which indicates that the intradermal vaccinations had considerable effect. Thus, 7 of 166 participants (4%) who completed a clinical questionnaire had symptoms comparable with influenza. This is in contrast to 28 cases with similar symptoms among 199 non-vaccinated family contacts (14%). Ratio of the attack rates in non-vaccinated and vaccinated groups in the present study (3.7) is interestingly similar to the corresponding ratio (3.6) in the study of Sigurjonsson *et al.*(8) even though the attack rates in the latter were much higher.

Undesirable side reactions from vaccinations are factors which greatly affect acceptance of a vaccination program. Although the amount of vaccine injected affects reaction rate and severity of the reaction, other studies performed simultaneously with this study but using different vaccines clearly indicated that reaction rate and severity of reaction vary as much from vaccine lot to vaccine lot as they varied in this study from dose to dose.

Summary. The immune response elicited by 2 intradermal injections of 0.1 ml monovalent Asian A influenza vaccine administered 2 weeks apart was related to the antigenic potency of the vaccine. Individuals developing detectable antibodies 2 weeks after the second injection varied with dose from 44 to 70% with titers 1:40 or greater. Using the same lot and dosage schedule of vaccine an intradermal booster immunization administered 53 weeks after first vaccinations, induced marked rise in titers which were also related to dose. The geometric mean titers which ranged from 1:21 to 1:51 two weeks after second injection of vaccine rose to 1:51 to 1:211, 2 weeks after booster injection. In the present study, 2 separate injections of 50 CCA units resulted in better antibody responses than single injections of 100 CCA units. Even the response to 2 injections of 12.5 CCA units is as good as the response to single doses 4 times as large. Systemic and local reactions increased with unit dose of vaccine but no serious reactions occurred with the lot of vaccine used throughout this study.

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In vitro Metabolism of Mephenesin, 3-o-Tolyloxy-1,2-Propanediol. (25443)

ALFRED R. MAASS, PAUL L. CAREY,* ROBERT E. HAMILTON,† AND ARTHUR E. HEMING
Smith Kline & French Labs., Philadelphia, Pa.

3-o-Tolyloxy-1,2-propanediol, mephenesin, a muscle relaxant, is characteried by an extremely short duration of action. Two metabolic products, 3-(o-tolyloxy)lactic acid(1,2) and 3 - (4-hydroxy-2 - methylphenoxy)lactic acid(3,4,5) have been identified in dog and human urine following administration of mephenesin. Aside from these *in vivo* observations, *in vitro* metabolism of this compound has not been reported beyond the brief comment of Maass, *et al.*(6). The metabolism of mephenesin by mouse and rat liver slices has

* Present address, Ralston Purina Co., St. Louis, Mo.

† Present address, Western Reserve Univ., Cleveland, O.

TABLE I. Mephenesin Metabolism in 60 Min. by Rat and Mouse Liver Slices.

Animal	No. of exp.	Buffer	Mephenesin metabolized (by difference)		3-(o-tolyloxy)lactic acid formed,
			μmole	μmole/g ± S.D.	μmoles
Rat	2	K.R. phosphate	3.41	7.91	2.94
Mouse	5	"	3.59	9.14 ± 2.63	1.63
Rat	2	K.R. bicarbonate	2.50	6.24	1.31
Mouse	5	"	5.64	14.58 ± 3.22	2.81

been studied under various experimental conditions and the principal metabolite has been identified and assayed.

Methods. Approximately 400 mg fresh weight of liver from adult rats or mice, usually 5 or 6 slices, were incubated with 11 μM mephenesin in 20 ml Krebs-Ringer (K.R.) phosphate (pH 7.4) or bicarbonate buffer (pH 7.4) in a 125 ml Warburg flask. The flasks were incubated for 30 to 180 minutes at 38°C with either 100% oxygen or 95% oxygen and 5% carbon dioxide. Phthalate, ethylene diamine, or borate buffers at pH 6.1, 7.4 and 8.1 were also tried but the reaction proceeded more rapidly in K.R. phosphate or bicarbonate buffer. The reaction was stopped by filtering the medium rapidly through Whatman No. 41 filter paper to separate medium and tissue. Trichloroacetic acid, which might have been used to stop the reaction, was found to interfere with the ultraviolet assay of the substrate and product, therefore, the filtration procedure was devised. After incubation the medium was routinely assayed for both mephenesin and 3-(o-tolyloxy)lactic acid(7). Mephenesin was exhaustively extracted from the incubation medium (pH 6.0-8.1) with ether. The medium was then adjusted to below pH 3.0 in order to suppress the ionization of the 3-(o-tolyloxy)lactic acid, which then was also readily extracted with ether. Substrate and product present were determined by recording the absorbance of the ether solutions between 240-300 mμ. The quantity of mephenesin or 3-(o-tolyloxy)lactic acid present was calculated from the absorbance at 270 mμ. The lack of absorbance at 290 mμ demonstrated the absence of any detectable quantity of 3-(4-hydroxy-2-methylphenoxy)lactic acid(4). To prove the identity of 3-(o-tolyloxy)lactic acid, the pooled ether extract was concentrated to 10 ml, then shaken gently with 10 ml of 0.1 N NaOH. Both layers were assayed spectrophotometrically at 300 and 270 mμ. Since 3-(o-tolyloxy)lactic acid is more readily soluble in NaOH than in ether, the presence of the characteristic ultraviolet absorption spectrum in the NaOH and the absence of absorption in the ether served to identify the reaction product. Mephenesin has a partition coefficient of 4.155 ± .093 in this system. Recovery of mephenesin, incubated with heat inactivated liver slices averaged 101.0% with a standard deviation of 5.7%. A small and variable amount (2.3 to 6.2%) of the mephenesin in the medium adhered to the tissue slice. Therefore, the tissue as well as the medium was assayed for mephenesin. Recovery of 3-(o-tolyloxy)lactic acid averaged 102.8 ± 3.2% in bicarbonate buffer and 106.1 ± 3.0% in phosphate buffer. There was no apparent tissue binding of the 3-(o-tolyloxy) lactic acid.

Results. A heat labile enzyme system present in rat or mouse liver slices rapidly oxidized mephenesin to 3-(o-tolyloxy)lactic acid (Table I and Fig. 1). Rat or mouse liver homogenates, supplemented with various cofactors under these conditions, did not cause oxidation of mephenesin. No metabolism occurred in presence of 100% nitrogen.

Representative data showing the relationship of mephenesin oxidation to tissue weight, substrate concentration and incubation time have been indicated in Figs. 1-3. The μmoles of mephenesin metabolized was directly related to amount of enzyme present (Fig. 1) but amount of 3-(o-tolyloxy)lactic acid formed was not proportional to tissue weight. The μmoles of mephenesin metabolized per gram of fresh tissue was apparently a linear relationship with time up to 60 minutes (Fig. 2).

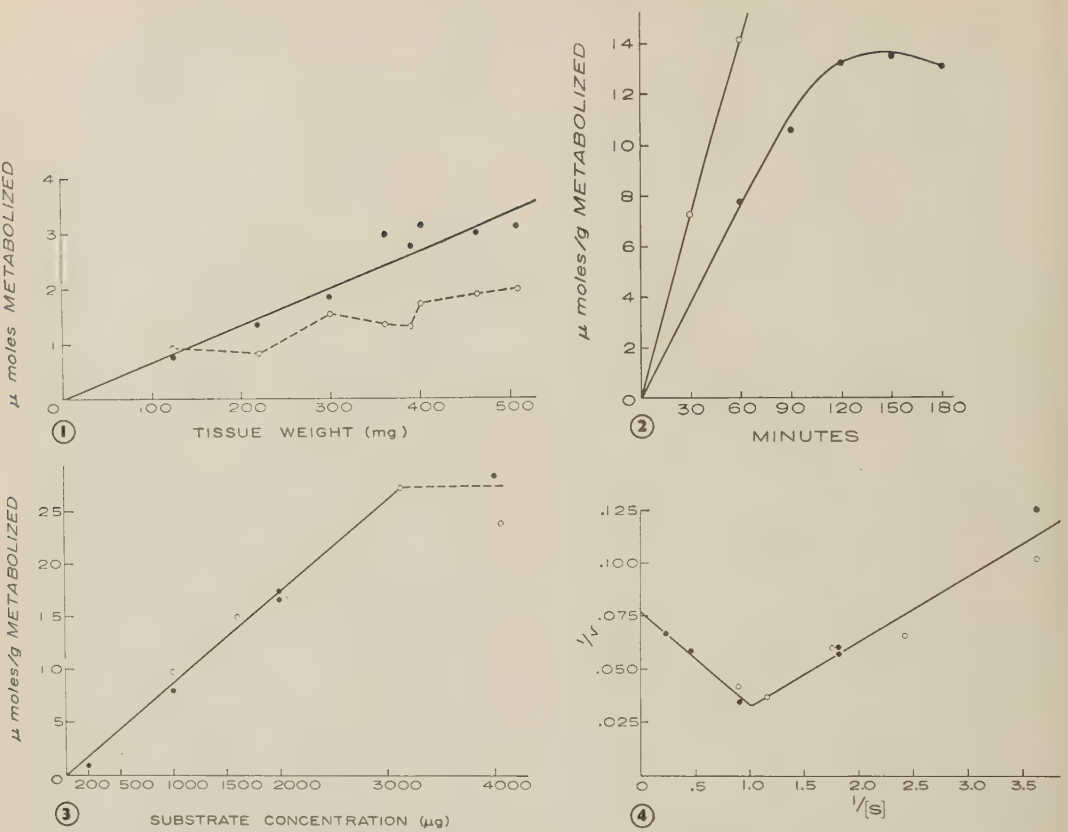

FIG. 1. μmoles of mephenesin oxidized (●) and 3-(o-tolyloxy)lactic acid (○) formed by increasing weight of mouse liver slices incubated in K.R. phosphate buffer (pH 7.4) for 60 min.

FIG. 2. μmoles of mephenesin oxidized by rat liver slices (●) in K.R. phosphate buffer (pH 7.4) or by mouse liver slices (○) in K.R. bicarbonate buffer (pH 7.4) between 30 and 180 min.

FIG. 3. Effect of substrate concentration on the rate of oxidation of mephenesin. Mouse liver slices incubated for 30 min. (○) or 60 min. (●) in K.R. bicarbonate buffer (pH 7.4).

FIG. 4. Effect of increasing substrate concentration [s] on observed velocity (v) of mephenesin oxidation. Mouse liver slices incubated for 30 min. (○) or 60 min. (●) in K.R. bicarbonate buffer (pH 7.4) containing 0.27 to 4.39 mM of mephenesin. Since reaction rate was linear for at least 60 min. the μmoles of mephenesin oxidized/g of fresh tissue/30 min. was doubled in calculating reciprocal of velocity.

The optimum substrate concentration was determined by incubating varying concentrations of mephenesin in bicarbonate buffer with approximately 400 mg of mouse liver slices (Fig. 3). The oxidation was depressed at low substrate concentrations, presumably due to a dilution effect. The maximum percent of substrate oxidized occurred between 1500-2000 μg. However, since rate of oxidation appeared to fall with increasing substrate concentrations, the data were extended and a Lineweaver and Burk(8) plot of the data (Fig. 4) clearly indicated inhibition of oxidation at concentrations above 200 γ/ml suggesting that the enzyme has 2 points of attachment for the substrate. It is obvious that mephenesin was not completely converted to 3-(o-tolyloxy)lactic acid (Table I). This suggested that either the 3-(o-tolyloxy)lactic acid was being further metabolized or that an intermediate metabolite between the alcohol and the acid was being formed. Although a number of conditions were tried none were found which would force the metabolism of 3-(o-tolyloxy)lactic acid. Addition of increasing amounts of 3-(o-tolyloxy)lactic acid to the medium actively oxidizing mephenesin had only a slight effect on rate of mephenesin metabolism (Table II). However, since there was a marked reduction in amount of 3-(o-

TABLE II. Effect of Increasing Amounts of 3-(o-tolyloxy)lactic Acid on Metabolism of Mephenesin.

3-(o-tolyloxy)-lactic acid, μmoles added	Mephenesin metabolized* (by difference) μmoles	μmoles/g	3-(o-tolyloxy)-lactic acid formed, μmoles
0	5.88	16.59	3.40
1.61	5.76	16.07	3.23
5.29	6.15	16.14	2.47
10.47	5.65	15.21	1.58
20.93	5.80	14.79	.13
41.88	5.36	14.29	.0

* K.R. bicarbonate buffer, 60 min. incubation.

tolyloxy)lactic acid formed, these data suggested that an intermediate metabolite was formed and that 3-(o-tolyloxy)lactic acid was not the immediate but the final product of mephenesin metabolism.

Since an aldehyde seemed to be the most likely intermediate, mouse liver slices were incubated with aldehyde trapping agents. Addition of either sodium bisulfite, hydroxylamine or semicarbazide at 10^{-4} and 10^{-3} M concentrations, which had no effect on oxygen consumption of the slices, had no effect on rate or extent of mephenesin oxidation, nor did they significantly alter amount of 3-(o-tolyloxy)lactic acid formed.

Alcohol and aldehyde dehydrogenases have been shown to require intact sulfhydryl groups for activity. Addition of iodoacetic acid to the medium indicated that mephenesin metabolism was sensitive to sulfhydryl inhibitors, for the oxidation was completely inhibited by concentrations as low as 3 mM. Preliminary studies indicated that mephenesin was not metabolized by mouse diaphragm, heart, spleen, or brain slices. Kidney slices oxidized mephenesin slowly.

Berger(9) reported that the LD_{50} for mephenesin, administered intravenously, increased from 195 mg/kg for the rat, to 220 mg/kg for the rabbit, to 322 mg/kg for the mouse. These data suggest that the mouse metabolizes the drug more rapidly than the rat. Our observations on the rapidity of mephenesin oxidation by the rat and the mouse support this conclusion.

Summary. 1. Mephenesin is oxidatively metabolized *in vitro* by rat and mouse liver slices to 3-(o-tolyloxy)lactic acid. 2. Mephenesin metabolism was markedly inhibited by increasing substrate concentration, but was not altered by presence of increasing concentrations of the product, 3-(o-tolyloxy)lactic acid, in the medium. 3. Metabolism of mephenesin was readily inhibited by iodoacetic acid, but was unaltered by aldehyde trapping agents at 10^{-3} M.

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Preparation of Formalinized Erythrocytes. (25444)

LAJOS CSIZMAS (Introduced by Margaret Pittman)
Division of Biologics Standards, N.I.H., P.H.S., U. S. Dept. of Health, Bethesda, Md.

Following discovery by Flick(1) that red blood cells treated with formaldehyde could be preserved for long periods of time, several investigators(2-4) devised methods of preparing formalinized cell reagents in which special properties of red cells were utilized(5). We prepared formalinized red cells by published methods which were not satisfactory for use in agglutination tests by the pattern method (6), because of crenation, inseparable clumps, and non-specific aggregation. Then we devised a method that permitted us to prepare con-

sistently, formalinized red blood cell suspensions of the desired quality. Cells thus prepared have been distributed and used successfully for various purposes(7). Although some improvements in technic have been described(8-11), a general analysis of the factors affecting formalinization is lacking. This paper describes our method and discusses the principles upon which it was designed.

Materials and methods. Bloods collected through a system thoroughly wetted by and into modified Alsever's solution(12) were stored at 4°C up to 4 weeks before use. Processing the cells within a few days after collection was advantageous because fresh cells were less fragile. On day of formalinization the cells were separated (for example from 100 ml of blood) by centrifugation at 600 to 1000 g and washed 5 to 7 times in 10 volumes (500 ml) of cold 0.85% NaCl solution. During washing and at subsequent stages of procedures, care was taken to avoid foaming of the liquid. The washed packed cells (50 ml) were resuspended in not less than 8 volumes (400 ml) of phosphate-buffered saline solution (0.009 M Na_2HPO_4, 0.006 M KH_2PO_4, 0.135 M NaCl) at pH 6.8 to 6.9. Formaldehyde solution, 40%, U.S.P., pH 5.5 to 6.0, equal to ¼ (100 ml) of volume of cell suspension, was poured into a cellophane dialysis sac (flat width 2.5 cm), twisted and knotted at one end. The tubing was cut off so that about one-third of sac remained empty. The air was expelled and the open end tied off. This sac was placed on bottom of beaker, and the cell suspension poured over it. The beaker was agitated at room temperature on mechanically operated orbital shaker. Speed of shaker was adjusted to provide most vigorous mixing action attainable without causing more than a small amount of foam. After 2 hours, the cellophane sac was removed and its contents poured into the beaker. Shaking was continued for additional 12 to 18 hours. Then a mass of clotted cell debris was usually found floating on surface of liquid or adhering to walls of beaker. With care in decanting the suspension into a clean container or filtering it through surgical gauze, it was not difficult to exclude all debris. To the homogenous cell suspension was added ½ volume (250 ml) of saline, then cells were washed 6 times in about 750 ml. More meticulous washing seemed unnecessary, as our observation in agreement with Cox(3) indicated that some formalin may be added without causing further change in the cells; a repeated formalinization had no detectable effect on the cells. Finally, the packed formalinized cells (about 60 ml) were suspended in an equal volume of saline, with or without 0.02% thimerosal, and stored as 50% stock suspension at 4°C.

Results. After many trials with changes and variations in technics, the above method was selected. It has been used satisfactorily in preparation of red cells from 7 species of animals—human, bovine, rabbit, guinea pig, chicken, alligator and sheep. With the latter, however, it was desirable to use 0.85% NaCl solution instead of phosphate-buffered saline solution. With this minor modification, 24 successful preparations have been obtained.

The final cell suspensions typically were brown, changing to reddish-brown upon dilution. For a few days after dispersion, the cells were readily redispersed by gentle shaking. After a longer period, more thorough shaking was necessary to obtain a uniform suspension, but the cells showed comparatively little tendency to stick to walls of vessel or to aggregate nonspecifically. When examined microscopically (Fig. 1a), suspensions contained individual cells (not more than 2% of cells were aggregated), which were slightly larger and more nearly spherical in shape than untreated cells of the same species. The smooth convex periphery of treated cells is illustrated in Fig. 2.

After formalinization, these cells were not subject to hemolysis in hypotonic salt solutions or in presence of complement and specific antibody. Their resistance to mechanical injury was excellent, and they sustained no discernible change in properties after storage for one year or longer at 4°C.

The sharp endpoints and distinct negative patterns of which these cells are capable, are illustrated in the hemagglutination test shown in Fig. 3. Sheep erythrocytes sensitized with

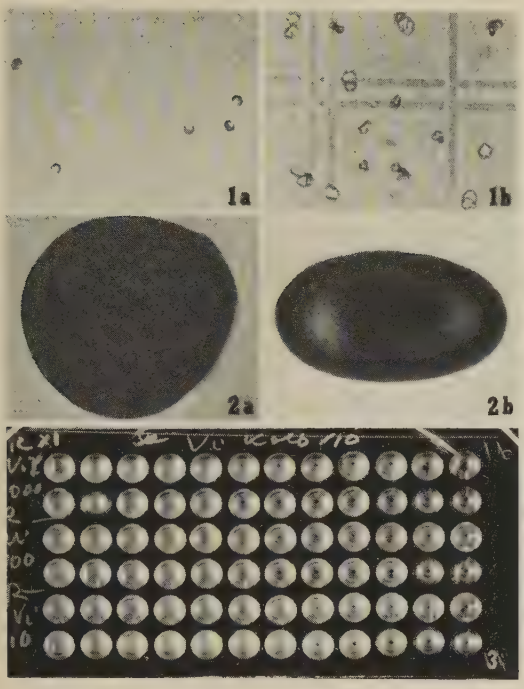

FIG. 1. Formalinized red blood cells prepared by present method (a); and according to published methods(1-4), pool of 4 preparations (b).

FIG. 2. Electron micrographs of a formalinized sheep (a) and chick (b) red blood cell.

FIG. 3. Hemagglutination on Takatsy plate using anti-Vi serum and Vi sensitized formalinized sheep cells.

Vi antigen were used in this experiment in a modification of the Takatsy plate method (13). Two-fold serial dilutions of Vi antibody were made in saline solution (0.025 ml) and the sensitized formalin-treated erythrocytes (0.025 ml of a 0.33% suspension) were added. Homogeneity of treated cells is indicated by sharpness of endpoint. In the second line a double amount of cells (0.05 ml) was used with no change in endpoint. With higher concentrations of Vi antigen, shown in top lines, the titer decreased and negative patterns were more distinct because of the agglutinative protective property of the Vi antigen. This conforms with previous findings(14).

Formalinized cells prepared from blood from different species of animals have been successfully sensitized with Vi antigen. In addition, tannin treated or diazotized formalinized cells have been sensitized with diphtheria and tetanus toxoids, respectively, and sheep cells so treated are used routinely in a study to be published.

Discussion. To produce distinct negative patterns in hemagglutination tests, suspensions of erythrocytes must be not only relatively free of aggregated cells but the individual cells also must have those properties which favor their collection into a compact button-shaped mass at the bottom. Ideally, the cells should be spherical, with smooth surfaces that are not sticky and do not adhere to wetted glass. To obtain formalinized cells with these qualities the following principles were applied. (1) Thorough washing of cells, prior to addition of formaldehyde, avoids stickiness which can result from gelling of plasma proteins on cell surface(15). Further, an hemolysate, free of cells treated with formalin, would readily gel or form clumps. Hence excessive washing which increases cell fragility and hemolysis is to be avoided. (2) High dilution during formalinization decreases concentration of contaminating plasma or hemolysate, and reduces the chances for cell contacts which influence aggregation and distortion. (3) Shaking distributes cells uniformly and helps to eliminate gel forming contaminants by promoting formation of a discrete pellet. Excessively vigorous agitation is disadvantageous, particularly if considerable foam appears, since deformation or lysis of cells may occur at air-water interface. (4) Slow addition of formaldehyde solution by dialysis permits counteractions to take place. Increasing concentration of formalin tends to cause crenation of cells, while declining pH and osmolarity cause cells to swell and assume smooth rounded shapes favorable to subsequent use in hemagglutination tests. On the other hand these factors increase the probability of hemolysis. The method described represents a balance between contesting requirements.

Summary. An improved method for formalinization of erythrocytes from different species of animals is described. Reproducible preparations, relatively free from crenation and non-specific agglutinability, are satisfactory for various agglutination tests by the pattern method, and may be stored for one year or longer at 4°C without loss of capacity

to react in these tests. The principles applied to attain satisfactory formalinized cells are discussed.

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Instability of Metabolic Quotients Obtained from Tissue Cultures.* (25445)

H. J. PHILLIPS AND R. V. ANDREWS
Dept. of Physiology and Pharmacology, Creighton University School of Medicine, Omaha, Neb.

To compare the metabolism of one tissue culture with that of another they should be maintained under conditions that yield reproducible results. Serum in tissue culture medium introduces a variable. Since serum is not chemically defined, much work has been done to devise synthetic growth media. Some tissue cultures have been grown in absence of serum(3,4) but these conditions are the exception rather than the rule, because most cell strains must still be maintained in serum. Although use of serum in nutritional studies can be questioned, there has been little objection to its use for growing cells for subsequent metabolic determinations, especially if cells are washed and resuspended in a chemically defined solution before the determination is made. We noted previously(7) that HeLa cells grown in medium containing human serum respired and formed lactate at a greater rate than the same cells grown with horse serum. These observations suggested the possibility that tissue cells grown in serum from different members of the same species may respire and form lactate at different rates.

Materials and methods. Earle's Strain L cells were grown in saline solution containing 0.5% enzymatic lactalbumin hydrolysate plus 20% horse serum. Cultures were gassed with 5% CO_2 in air. Saline solution contained in g/liter: NaCl 6.5, KCl 0.29, $MgSO_4 \cdot 7H_2O$ 0.21, $CaCl_2$ 0.14, $NaHCO_3$ 2.06, Na_2HPO_4 0.15, glucose 2, phenol red 0.05, lactalbumin 5. This and other solutions used, with the exception of trypsin, contained inorganic salts in about the same ratio as in interstitial fluid (12) and had a tonicity equivalent to 0.93% sodium chloride solution as determined by freezing point depression. All solutions were buffered at pH 7.2. At the start of experimentation, cells were grown in 1.9, 7.5, 15, 20, and 30% horse serum to determine optimal serum concentration for growth. Media was renewed every third day and after 10 days the live cells in each culture counted(8). These results graphed in Fig. 1 indicate that 15-20% horse serum plus lactalbumin medium was optimal for growth of Strain L cells. To prepare cells for metabolic determination, they were freed from T-60 culture flasks(2) with

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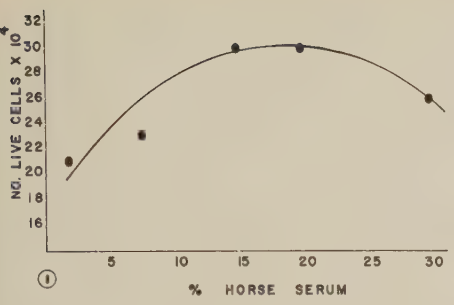

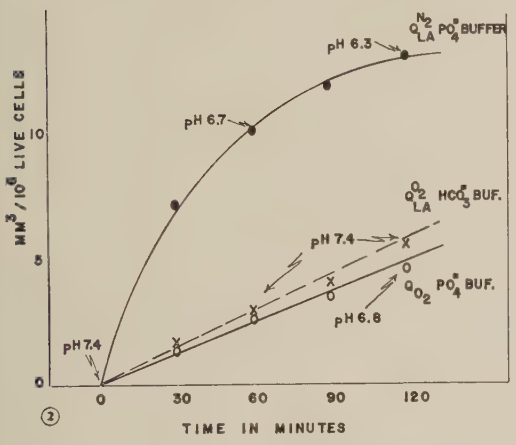

FIG. 1. Effect of horse serum concentration on growth of Strain L cells. Each point represents mean population of 4 roller tube cultures.

FIG. 2. Effect of pH on rate of lactate formation and oxygen uptake. $Q^{N_2}_{LA}$ and $Q^{O_2}_{LA}$ are graphed so that lines do not overlap; rate of both decreases as pH becomes lower.

trypsin on day following renewal of culture medium. The trypsin solution contained 2.5 g trypsin (Nutritional Biochemical Corp. 1:300) in one liter of 0.9% NaCl, and was buffered with 0.29 g Na_2HPO_4 and 0.04 g $NaH_2PO_4 \cdot H_2O$. Cells were dispersed by passing them back and forth through a syringe. They were then collected by centrifugation and half of the cells resuspended in a bicarbonate buffered solution and half in one buffered with phosphate. The bicarbonate solution contained in g/liter: NaCl 7.03, KCl 0.29, $MgSO_4 \cdot 7H_2O$ 0.21, glucose 2, $CaCl_2$ 0.14, Na_2HPO_4 0.15, $NaHCO_3$ 2.06 and gelatin 5. The phosphate solution contained: NaCl 8.42, KCl 0.29, $MgSO_4 \cdot 7H_2O$ 0.21, glucose 2, $NaH_2PO_4 \cdot H_2O$ 0.04, $CaCl_2$ 0.14, Na_2HPO_4 0.31 and gelatin 5. Gelatin was added as a protective agent to each solution because it yielded about 25% more live cells(5) than saline alone. The bicarbonate solution was used to suspend cells for determining lactate. Reaction vessels were gassed with 5% CO_2, either in air or in nitrogen. Lactate was determined chemically as previously described(9). Under these conditions pH 7.2 was held constant which was necessary because it had been found that lactate formation varied greatly with slight changes in pH(9). Respiration was not affected by slight changes of pH as long as pH remained within a range not harmful to tissue cultured cells(8). Results that illustrate the effect of pH change on glycolysis and respiration are graphed in Fig. 2. As pH in phosphate buffered saline decreased due to acid formation, rate of lactate formation also decreased; however, if pH was held constant with strong bicarbonate buffer, lactate formation continued as a straight line function with time. Rate of oxygen uptake in phosphate buffered saline did not change even though pH decreased. As a result of these observations glycolysis was measured in bicarbonate and respiration in phosphate buffered saline in an atmosphere of air by the Direct Method of Warburg(10). All determinations on the Warburg respirometer were made at 37.5°C and metabolic values expressed in terms of live cell counts, made as previously described(8).

Results. Strain L cells which had been grown for 30 days in medium containing horse serum A, had a stable rate of respiration and lactate formation. These results are shown in Table I for 30th and 31st day of experiment. On 32nd day, the cells were changed to a medium containing horse serum B. After the cells had been grown in this serum for 19 days, lactate formation in air was greatly increased; aerobic glycolysis was about the same as anerobic glycolysies. Thirty days after cells had been in horse serum B, respiration and lactate formation stabilized, but at a rate higher than observed in horse serum A. Starting on 67th day the experiment was repeated by growing cells in serum C and similar results were obtained.

These results indicate that respiration and glycolysis of tissue cultured cells may change when culture medium serum is changed. This

TABLE I. Change of Strain L Cell Metabolism with Change in Culture Media Horse Serum.

Day of exp.	Serum in medium	Amt (l)	Days in serum	Q_{O_2}	$Q^{O_2}_{LA}$	$Q^{N_2}_{LA}$
30	A	9-10	30	1.4	4.6	12.8
31	A		31	1.6	3.1	10.9
50	B	8	19	1.4	18.7	20.7
61	B		30	2.7	8.1	17.1
66	B		35	2.8	9.5	23.2
81	C	4	15	.5	18.3	19.6
97	C		31	3.5	10.0	23.5
98	C		32	3.7	11.0	27.2

Q = $mm^3/10^6$ live cells/hr. Q_{O_2}—oxygen uptake, mean of 12 determinations. Q_{LA}—lactate, mean of 4 determinations.

occurs even though serum is from the same species of animal, and when cells are washed and resuspended in saline before a metabolic determination is made. A change in metabolism by changing culture serum, is first reflected by an increase in aerobic lactate formation which is out of proportion to respiration or anerobic lactate formation. After the cells have been grown in a particular serum for about 30 days, adaptation appears to occur and metabolism stabilizes at a new level.

Cells had to be grown in a particular serum to change the metabolism. This can be shown in another way than that described above. Cells grown in serum C respired for several hours at the same rate even after they were washed and resuspended in either saline, serum C or serum A (Table II). These results also indicate that respiration, as measured, is not immediately affected by possible metabolite differences of the 2 sera.

Rate of oxygen uptake is related to growth rate. If growth rate is lowered by reducing incubator growth temperature to 31°C, respiration is subsequently lowered (Table II). That is, respiration is lower even though actual determinations were made at 37.5° in phosphate saline. Similar effects can be obtained by changing the quality of serum. A greater percentage of serum is collected from blood if an animal is bled excessively. Simply stated, blood becomes more dilute as hemorrhage progresses, which is one of the physiological mechanisms for maintaining blood volume. As an example, serum B was obtained from a horse bled 8 liters. It was lighter in color and contained less solids than serum from a horse bled 4 liters (serum C). The latter serum appeared to contain more nutrients since it stimulated growth of cells better than the former. Metabolism was also higher for cells grown in the more concentrated serum (serum C) than for those grown in serum B as indicated in Table I. A similar effect appears to occur by lowering culture serum concentration from 20 to 10%, Fig. 2. Respiration was higher for cells grown in 20% serum than for those grown in 10%. Actually the values are not statistically different. The difference ($P<.08$) in respiration falls just below the arbitrary 5% level of significance and for this reason are considered indicative rather than factual.

Discussion. There appear to be 2 mechanisms which could explain the results in our experiments. Young or rapidly dividing cells have a metabolic rate different from that of old cells. Culture conditions which alter growth rate can also alter the ratio between young and old cells and therefore alter the metabolic picture. Our results and observations support this conclusion. Other evidence comes from an earlier report(6), in which we found that Strain L cells had a higher rate of oxygen uptake, if kept in a rapidly dividing state as compared to an old culture. Respiration of cultures fed the day before a Warburg test was twice that of cultures which had not been fed for 5 days.

When cells are transferred from one serum to another, aerobic lactate formation rises to a level which is out of proportion to oxygen uptake or anerobic lactate. Adaptation to the medium appears to occur in about 30

TABLE II. Effect of Growth Temperature or Serum on Respiration of Strain L Cells.

Serum in medium	Warburg solution	Growth temp.	Q_{O_2}	P
20%—C	Saline	37	3.3	
"	" + 10% serum A	"	3.5	
"	" + 10% " C	"	3.3	
20%—D	"	"	3.2	<.01
"	"	31	2.2	
20%—D	"	37	3.1	<.08
10%—D	"	"	2.8	

days and aerobic lactate returns to a moderate level. An explanation for this reaction or mechanism is unknown.

Our results introduce several important problems. If one wants to compare metabolism of one strain of tissue cultured cells with that of another they should be grown under the same conditions and in the same medium. The same applies if one compared metabolism of a normal tissue cultured cell to that of a tumor or virus infected one. Metabolic quotients for respiration or glycolysis of tissue cultured cells when considered alone, have little quantitative significance. These values cannot be duplicated precisely because sera from various laboratories will differ.

Although the exact nature of serum may not be known, various cell strains can be grown in a particular serum under identical conditions for a limited time, to compare metabolic rates. Cells derived from either normal or malignant tissue can thus be grown in tissue culture systems at a similar rate. Tissue cultured cell strains derived from normal or malignant tissue, in the same medium, growing at similar rate, have the same rate of respiration and lactate formation(1). Is it possible that respiration and glycolysis of a tumor differ basically from that of a normal tissue or could it be that the difference is due to a dominance of rapidly growing young cells? There is evidence that rapidly growing tissue such as bone marrow (11) and embryonic tissue, even when taken directly from the host, presents a metabolic picture similar to that associated with tumors.

Summary. Maximal growth of Earle's Strain L cells is obtained by using 15-20% horse serum with a medium containing 0.5% lactalbumin hydrolysate. Rate of lactate formation decreases as pH decreases but remains constant if the suspension is adequately buffered with bicarbonate. Respiration continues as a straight line function with time even though pH decreases. Sera from different horses affects growth rate of tissue cultures differently and as a consequence a culture in one horse serum may contain more rapidly dividing cells than another culture in another horse serum. Young, rapidly growing tissue cultured cell strains respire and glycolyze at a greater rate than old ones. This difference in metabolic rate occurs even though the cells are washed free of culture medium and resuspended in balanced saline solution before the metabolic determination is made. Metabolic rate is related to growth rate. This can be easily demonstrated by simply growing the cells at 31°C even though the actual determination is made at 37.5°C. Metabolic quotients obtained on tissue cultures grown in serum, have little quantitative significance when considered alone, because serum is not chemically definable.

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Isolation and Characterization of Prototype Viruses ECHO-26, ECHO-27, Coxsackie B-6.*† (25446)

WILLIAM McD. HAMMON, DAVID S. YOHN AND ROSE A. PAVIA‡
Dept. of Epidemiology and Microbiology, Graduate School of Public Health, Univ. of Pittsburgh

In December 1955, the Committee on ECHO Viruses of the National Foundation described common properties of the first 13 ECHO prototype viruses(1). Subsequently the scope of the Committee was expanded to include Coxsackie and polioviruses and was renamed Committee on Enteroviruses(2). This Committee presently recognizes 3 types of poliovirus, 28 types of ECHO viruses, and 6 types of Coxsackie B. As yet no action has been taken on Coxsackie A group. This report describes the recently accepted enterovirus prototypes, ECHO-26 and 27 and Coxsackie B-6, which we isolated from rectal swabs collected in the Philippine Islands during a longitudinal epidemiological study of enteroviruses at Clark Air Force Base (CAFB), Luzon, in 1953(3).

Materials and methods. 1. *Sources.* Essential details of source of each prototype virus are presented in Table I. All came from normal persons, 2 of whom were children, and 1 an adult; 2 were Americans and 1 was a Filipino. All viruses were isolated from rectal swabs collected in 1953. 2. *Cell culture.* Trypsinized rhesus monkey kidney cell cultures (RhMKCC) were grown in 0.5% lactalbumin hydrolysate in Hank's (LAH) balanced salt solution (BSS) containing 2% calf serum. Prior to use in neutralization tests the cells were changed to 0.5% lactalbumin hydrolysate in Earle's BSS (LAE) containing 0.5% calf serum. For preparation of stock virus pools for immunization of animals and for use in complement fixation (CF) tests, the cells were changed to Eagle's Basal Medium (EBM) in Earle's BSS without serum. Hamster kidney was grown in LAH with 4% calf serum according to method of Diercks and Hammon(4). The transplantable cell lines HeLa (Gey)§, HeLa (Puck S-3),‖ H. Ep. #2,¶ and K.B.** were carried in EBM with 10 to 20% pooled human serum or 10% calf serum. Cytopathogenic tests with these cell lines were conducted in EBM containing 5% inactivated calf serum. 3. *Plaque methods.* RhMKCC grown in 3 oz. bottles, described by Hsiung and Melnick(5), were used in plaque studies. Occasionally, for comparative studies, the final $NaHCO_3$ content was reduced from 3 ml to 1.25 ml of a 7.5% solution/100 ml of overlay. This modified overlay also contained 10 ml of 3% Tris buffer solution (pH 7.4)/100 ml. The final agar concentration was also occasionally reduced from 1.5 to 1%. 4. *Purification of virus.* Each of the 3 viruses was purified by either the plaque picking method (3 serial passages) or, if the agent did not form plaques, by terminal dilution (3 serial pasages). These "purified" agents were used for immunization of animals and as seed virus in all serological tests for typing. 5. *Production of antisera.* "Purified" virus seed grown in RhMKCC in EBM without serum was harvested, extracted once with equal volume of fluorocarbon(6), lyophilized and reconstituted to 1/10 original volume. Two ml of this material and 2 ml of Arlacel-Bayol F adjuvant were emulsified and injected intramuscularly into monkeys. Injections of this same material and same dose were given at 7 to 14 day intervals for 3 to 5 months, length of time being dependent on titer of test bleedings. 6. *Serologic tests.* Neutralizing antibody (NA) tests were performed as described previously, using serial

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‡ The authors wish to express appreciation to Della M. Whitaker for technical assistance.

§ Cell line obtained from Microbiological Assoc., Washington, D.C.

¶ Cell line obtained from Dr. Jonas Salk, Pittsburgh, Pa.

** Cell line obtained from Dr. Joseph Leighton, Pittsburgh, Pa.

TABLE I. Source, Method of Isolation and Certain General Characteristics of Prototype ECHO-26, ECHO-27 and Coxsackie B-6 Viruses.

	Virus		
	11-3-6* ECHO-26	1-36-4* ECHO-27	1-51-21* Coxsackie B-6
Human source	Coronel	Bacon	Schmitt
Age and sex	1, ♂	10, ♀	25, ♂
Race	Filipino	American	American
Location	Near CAFB,† Luzon	CAFB, Luzon	CAFB, Luzon
Disease	None	None	None
Specimen	Rectal swab	Rectal swab	Rectal swab
Date collected	9/17/53	8/17/53	11/24/53
Isolation(s) RhMKCC	4/ 1/55	12/16/55	10/13/55
Reisolation	Q.N.S.‡	12/20/57	Neg. (12/58)
Additional	None	None	8-Hammon feces 2-Hummeler feces‖
Type of CPE	Polio-like (complete)	Polio-like (complete)	Polio-like (complete)
Titer/0.1 ml¶	10^6	10^6	10^5
Passages	12	13	10
End point	4-6 days	7-9 days	4-6 days
Purification	Plaque§	Terminal dilution	Plaque
Plaque type	Small, hazy	None	Large, clear

* First 2 numbers identify group and individual, last number represents particular specimen of that individual.
† CAFB = Clark Air Force Base.
‡ Q.N.S. = Quantity not sufficient.
§ Plaques produced under Tris buffer overlay only.
‖ Personal communication from Dr. Klaus Hummeler, Children's Hospital, Phila., Pa.
¶ Pool used for identification studies.

serum dilutions and an estimated 100 $TCID_{50}$ inoculum of virus(7). CF tests were performed in 13 x 100 mm test tubes using 0.2 ml of each reagent. Virus for CF antigens was grown in RhMKCC in EBM without serum and routinely extracted once with fluorocarbon. Antisera were diluted 1:5, inactivated for 30 minutes at 60°C, adsorbed with equal volume of 10% RhMKCC suspension for 1 hour at 37°C, then centrifuged for 30 minutes at 2,000 rpm. Undiluted antigen and dilutions of antiserum plus guinea pig complement were incubated at 37°C for 1 hour; sensitized sheep red blood cells were added and the test incubated another hour at 37°C. The endpoint was taken as highest dilution of antiserum which showed less than 50% hemolysis in presence of 2 full units each of complement and of hemolysin. 7. *Animal pathogenicity tests.* Baby mice, 24 hours old or less, received either 0.01 ml of undiluted virus intracerebrally (i.c.) or 0.03 ml subcutaneously (s.c.). Each virus was tested by both routes. Blind passages in mice were made at least twice, using 20% mouse brain and carcass suspensions. Monkeys received 0.5 ml i.c. and some 0.1 ml intraspinally (i.s.); rabbits were given 0.5 ml i.c. and adult guinea pigs, hamsters, and mice 0.1 ml i.c. Seven to 8-day-old embryonated eggs were inoculated with 0.1 ml of undiluted virus into the yolk sac. 8. *Hemagglutination tests.* These were performed according to method of Goldfield *et al.*(8) at 4, 25 and 37°C, with both chicken and human "O" cells. 9. *Desoxycholate test.* This was a modification of the method of Theiler(9). Equal volumes of 1:500 sodium desoxycholate and undiluted virus were incubated 1 hour at 37°C and then titrated in 10-fold dilutions in RhMKCC. Results were compared with control titrations of virus treated in the same manner but without desoxycholate.

Results. 1. *Isolations.* Dates of isolation, results of reisolation attempts and the isolation of additional strains are presented in Table I. 2. *Common characteristics.* The following characteristics were common for the 3 viruses: (1) They produced a cytopathogenic effect (CPE) in RhMKCC similar to that produced by polioviruses. (2) No CPE was noted in HeLa-Gey, HeLa S-3, H. Ep. #2,

TABLE II. Neutralizing Antibody Tests in RhMKCC with ECHO-26, ECHO-27 and Coxsackie B-6 Viruses against Antisera to Known Enteroviruses.

Antisera	Source laboratory	Homologous N.A. titer	Virus – ECHO-26 $TCID_{50}$*	ECHO-26 Titer†	ECHO-27 $TCID_{50}$	ECHO-27 Titer	Coxsackie B-6 $TCID_{50}$	Coxsackie B-6 Titer
ECHO- 1 to 12	Wenner	1,700–65,000‡	32–100	<10	20–50	<10	10	<10
13	Hammon	40,000	50	"	20	"	"	"
14 to 20	Wenner	600–16,000	"	"	"	"	10–32	"
21	Enders	128	100	"	"	"	320	"
22 to 24	Sabin	1,000–10,000	"	"	"	"	"	"
25	Rosen	2,500	"	"	100	"	100	"
26	Hammon	7,500	"	*7,500*	20	"	50	"
27	"	40,000	"	<10	"	*40,000*	"	"
Coxsackie A-7, 9, 11, 13, 15, 18	Syverton	64–1,024	50	"	"	<10	10	"
B-1 to 5	"	128–1,024	"	"	"	"	10–32	"
Idem	Wenner	400–20,000	"	"	"	"	32	"
B-6	Hammon	15,000	100	"	"	"	320	*15,000*
Poliovirus 1, 2, 3	Wenner	>1,000	50	"	"	"	10	<10

* $TCID_{50}$ of virus used in test as determined by control titration.
† Reciprocal of serum dilution calculated to protect 50% of tubes inoculated.
‡ Homologous N.A. titer of ECHO-4 was 1:30 to 1:128; CF tests carried out in addition (see text).

K. B. and primary hamster kidney and cat kidney cell cultures. (3) They were not inactivated by desoxycholate. (4) Chicken and human "O" red blood cells were not agglutinated at 4, 25 and 37°C. (5) The 3 viruses passed gradocol membrane filters of 36 mμ porosity but were retained completely (Coxsackie B-6 and ECHO-27) or almost completely (ECHO-26) by a 28 mμ porosity filter.†† (6) No significant immunologic relationship to any other human enterovirus presently known to propagate in RhMKCC was established by reciprocal testing in NA tests (Tables II, III). Further exclusion of identity with ECHO-4 virus was demonstrated in reciprocal CF testing. (7) A human antiserum known to yield a group reaction in CF tests with adenoviruses did not react with these viruses. (8) Serologic rises and persistence of specific NA's at 1:8 to 1:32 serum dilution were demonstrated for each of the 3 viruses in human sera collected in the Philippines (Table IV).

3. *Specific characteristics.* (a) *Coxsackie B-6 virus (1-51-21)* produced paralysis, death and typical Coxsackie B pathology in suckling mice upon direct passage from RhMKCC. Dr. Klaus Hummeler of Children's Hospital of Philadelphia reports that he has recently isolated 2 strains directly in suckling mice from feces of children with aseptic meningitis (personal communication). These were identified by sera sent to him by this laboratory. Coxsackie B-6 killed embryonated eggs inoculated by the yolk sac in an irregular manner; however, the virus could not be passed serially in eggs. Chick embryo pathogenicity

†† Tests kindly performed by Dr. Albert Sabin, Cincinnati, O. and results made available to us.

TABLE III. Neutralizing Antibody Tests in RhMKCC with Antisera to ECHO-26, ECHO-27 and Coxsackie B-6 Viruses against Known Enteroviruses.

Virus	$TCID_{50}$ range	Monkey antisera ECHO-26	ECHO-27	Coxsackie B-6
ECHO-26	20	*7,500*	<5	<10
-27	100	<5	*40,000*	"
Coxsackie B-6	320	"	<5	*15,000*
ECHO-1 to 25	3.2–500	<10	<10	<10
Coxsackie A-7	10	<5	<5	*10**
A-9	50	"	"	<5
B-1	50–500	"	"	"
B-2	100		*20*†	
	200	<5		<5
B-3,4,5	16–160	"	<5	<10
Poliovirus 1, 2, 3	50–320	"	"	<5

* 20 units of this serum represent a 1:750 dilution—result with low dilution used not significant for typing.
† 20 units of this serum represent a 1:2,000 dilution—result with low dilution used not significant for typing.

TABLE IV. Neutralizing Antibody Tests in RhMKCC with Human Sera and Gamma Globulin against ECHO-26, ECHO-27 and Coxsackie B-6 Viruses.

Serum source	Virus 11-3-6 (ECHO-26)	1-36-4 (ECHO-27)	1-51-21 (Coxsackie B-6)
Original host	No serum	<4*–64	No serum
Other:			
With titer rise	<4–8		<4–128†
With persisting titer (1:8 or >) in serial bleedings of individuals possessing antibody	3	2	3
Gamma globulin: 1 lot American	<25	>50	<25
" Japanese	"	"	"

* Reciprocal of serum dilution calculated as 50% endpoint.

† Dr. Klaus Hummeler has obtained serologic rises in 2 patients with aseptic meningitis from whom were isolated agents identified as Coxsackie B-6 with antiserum supplied by this laboratory.

is compatible with results occasionally obtained with other Coxsackie viruses(10). Coxsackie B-6 virus did not produce CPE in several human cell lines, HeLa, K.B. and H. Ep. #2, known to support growth of certain strains of Coxsackie B-1 through 5(10). This characteristic, therefore, differentiates it from the first 5 Coxsackie B viruses. Three of 8 strains of Coxsackie B-6 isolated were neutralized by very low dilutions of Coxsackie B-1 antiserum while 5, including 1-51-21, were not. No antisera prepared against the new strains neutralized Coxsackie B-1 virus. Furthermore, a 1-51-21 CF antigen, which reacted with homologous antiserum at a dilution of 1:128, did not react with antisera to Coxsackie B-1 to 5, or to ECHO viruses 1-9, 11-20, 24-27, to all 3 polioviruses, and to Coxsackie A-9.‡‡

(b) *ECHO-26 virus* (*11-3-6*) produced no clinical illness in mice, hamsters, rabbits, guinea pigs or monkeys by routes previously described. Sections of the cord and brain of the monkey sacrificed 27 days after inoculation, revealed no significant pathology. An interesting characteristic of this virus is that it produces plaques under 1% agar overlay with low $NaHCO_3$ content but not under agar containing high $NaHCO_3$ (See Materials and methods). Under the former conditions very small hazy plaques with ragged edges are formed.

‡‡ A portion of these tests was kindly performed by Dr. Leon Rosen, Nat. Inst. of Allergy and Infect. Dis., U.S.P.H.S. and results made available to us.

(c) *ECHO-27 virus* (*1-36-4*) was not pathogenic for suckling baby mice and embryonated eggs. It did, however, produce questionable clinical signs of central nervous system involvement in one monkey inoculated i.c. and i.s. The monkey, which had been irritable and tremulous prior to inoculation, had a single convulsion 4 days after inoculation. The animal survived but was sacrificed on 27th day. Sections of brain and cord revealed no significant pathology except for a mononuclear inflammatory reaction involving a portion of the choroid plexus of the lateral and fourth ventricles. A 1:20 dilution of ECHO-27 antiserum with homologous titer of 1:40,000 neutralized 100 $TCID_{50}$ of Coxsackie B-2 virus (Table III). Such a reaction is not interpreted to indicate identity or even close antigenic relationship. Furthermore, prototype ECHO-27 virus, in addition to being nonpathogenic for suckling baby mice as mentioned previously, does not produce plaques in RhMKCC and does not produce CPE in human cell lines. These 3 characteristics further differentiate it from recognized strains of Coxsackie B-2.

Summary. Herein are described the antigenic specificity and biologic properties of 3 new prototype human enteroviruses, ECHO's 26 and 27 and Coxsackie B-6. All 3 fulfill the necessary criteria descriptive of their respective enterovirus group and have been assigned numbers by the Committee on Enteroviruses. All 3 were isolated from rectal swabs taken from healthy persons living in the Philippines in 1953.

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Perphenazine and Reserpine as Antiemetics for Staphylococcal Enterotoxin.* (25447)

H. SUGIYAMA, M. S. BERGDOLL AND R. G. WILKERSON
(Introduced by G. M. Dack)
Food Research Inst., University of Chicago

Vomiting is one of the most characteristic reactions in staphylococcal food poisoning. The only laboratory procedure now available for detection of the etiological agent, staphylococcal enterotoxin, is the provocation of emesis in monkeys or cats following suitable challenge(1). Recently a number of antiemetics have been reported effective against various types of emetic agents or which control vomiting in various disease states. No specific study seems to have been made on use of these agents as antiemetics against staphylococcal enterotoxin induced emesis. The present communication reports on effectiveness of 2 tranquilizing drugs in inhibiting emesis in monkeys fed enterotoxin. Antiemetics effective in dogs against one or more of the emetics whose site of action is the chemoreceptor trigger zone(2) were tested: chlorpromazine(3,4), perphenazine(5,6), and cyclizine lactate(7). Reserpine was included, although reports on its ability to protect against apomorphine induced vomiting in dogs are contradictory(7,8). Non-specific protection by depression of the medullary reticular emetic center was evaluated by determining change in sensitivity of monkeys to orally administered copper sulfate which evokes vomiting by peripheral action on the gastro-intestinal tract(9).

Materials and methods. Monkey feeding tests were essentially that previously described(10). *Macaca mulatta* were selected into groups of approximately equal sensitivities to enterotoxin. The paired groups whose response to the toxin was to be compared were alternated, wherever possible, as control and drug treated groups to compensate for variations in reactivities to enterotoxin. Complete control of this variable was impossible due to development of tolerance to the emetic. Each drug was tested in different sets of animals. Enterotoxins were partially purified preparations derived from S-6 and 196E strains of *Staphylococcus* and are antigenically different. Dosage was increased with each feeding of same animals to overcome development of immunity. For intravenous challenge an S-6 enterotoxin of high purity (90% purity) was used. Monkeys were injected intramuscularly with perphenazine (Trilafon) immediately before and with chlorpromazine (Thorazine) and cyclizine lactate (Marezine) 30 min before oral administration of enterotoxin. Since these animals seldom vomit within 1 hr of feeding enterotoxin, seda-

* This work supported by contributions from various companies and associations of food industries.

tion was established during the latent period. Reserpine (Serpasil) was injected intravenously about 18 hr preceding challenge with toxin. Vomiting in response to oral copper sulfate was used as a measure of depression of the vomiting center by the antiemetics. Fasted monkeys were fed 50 ml of $CuSO_4 \cdot 5H_2O$ solutions at 2-fold increments beginning at 80 mg and animals observed for vomiting for 120 min. None of 8 monkeys vomited to 80, about 60% to 160, and about 85% to 320 mg. The latter amount was used as threshold dose; animals not responding within 60 min at this level in control feedings were not used. The 320 mg threshold dose was, therefore, 2× the actual minimum dosage in about 60% of monkeys. This may not be too different with animals challenged with enterotoxin since the latency and number of vomiting episodes indicate that many control animals received more than a single threshold dose. Actual minimal emetic dose of copper sulfate was previously established for each animal in testing possible depression of the vomiting center by perphenazine. Dogs were also tried. Dogs of 5-12 kg body weight were injected intravenously with the highly purified enterotoxin. Animals were used only once and were observed for 5 hr. Perphenazine was injected intramuscularly immediately after intravenous injection of enterotoxin; chlorpromazine was administered intravenously about 30 min preceding challenge with enterotoxin.

Results. Treatment of monkeys with 1 mg/kg body weight of reserpine, intravenously, about 18 hr preceding intragastric challenge with enterotoxin resulted in 68% reduction of vomiting incidence (Table I). The emetic response was more severe in control animals; each reacting animal vomited 2.5 times/enterotoxin feeding, as compared to those in the drug treated group which vomited 1.9 times. The average prodromal interval for the drug treated group was 124 min as compared to the 99 min for controls. Reserpine injection immediately before feeding of enterotoxin had no protective effect. The delayed but long lasting protection against vomiting is in accord with depletion of serotonin (11) and catechol amines(12) from body storage sites by reserpine.

TABLE I. Antiemetic Activity of Reserpine (1 mg/kg, i.v., 18 Hr Previously) against Vomiting Provoked by Orally Administered Staphylococcal Enterotoxin and Copper Sulfate.

Enterotoxin strain	Emetic mg/monkey	No. vomiting/No. fed Control	Drug treated
S-6	.1	3/6	0/6
"	.25	4/6	2/6
196E	1.5	6/6	1/6
"	2.5	4/6	2/6
"	.75	2/6	1/6
Totals		19/30	6/30
$CuSO_4 \cdot 5H_2O$	320	10/10	9/10

Reserpine (1 mg/kg) did not materially affect the incidence of vomiting in animals challenged with threshold level of copper sulfate, although latency was increased from 31 to 50 min. Of the 19 enterotoxin fed animals that vomited in the control group, 15 responded in less than 120 min and all except one vomited within 150 min. Since toxin fed animals are observed for 5 hr, delayed vomiting, if it occurred, would have been observed; thus, it is unlikely that the antiemetic action of reserpine is due to depression of the vomiting center. On the other hand, depression of the vomiting center could partly explain the resistance to enterotoxin following 2.5 mg/kg of reserpine (control 20/42 *vs.* 2/41 drug treated). With this drug dosage only 2/9 monkeys given threshold dose of copper sulfate vomited and these only after a latent period of over 2 hr as compared with the average of 30 min in control feeding.

Perphenazine reduced vomiting incidence in enterotoxin challenged animals to 8% of that in controls (Table II). The minimal effective dose of the antiemetic is about 50 μg/kg since 25 μg/kg did not have any protective effect. Average number of vomiting episodes and latent periods of animals of control and drug treated groups which reacted, were not appreciably different. Perphenazine was effective even when administered after feeding enterotoxin; 50 μg/kg, intravenously, protected against the toxin fed 45 min previously.

Inhibition by perphenazine of vomiting in monkeys fed enterotoxin is not due to depression of the vomiting center. Perphenazine reduced the copper sulfate stimulated emesis by only 22% with an increase in latency from 30

TABLE II. Antiemetic Activity of Perphenazine (50 μg/kg) against Vomiting Provoked by Orally Administered Staphylococcal Enterotoxin and Copper Sulfate.

Emetic		No. vomiting/No. fed		
Enterotoxin strain	mg/monkey	Control	Drug treated	Perphenazine
S-6	2.0	2/6	0/6	Immediate*
"	10.0	3/6	1/6	"
"	.05	4/6	1/6	"
"	.5	4/6	0/6	45 min. after†
196E	5.0	5/6	0/6	Immediate
"	1.5	5/6	0/5	"
"	3.0	2/5	0/6	45 min. after
	Totals	25/41	2/41	
$CuSO_4 \cdot 5H_2O$	160–320	9/9	7/9	60 min. before‡

* Intramusc., immediately before feeding enterotoxin.
† Intrav., 45 min. after feeding enterotoxin.
‡ Intramusc., 60 min. before feeding each animal predetermined threshold dose.

to 39 min. The average prodromal period in animals of control group which vomited after enterotoxin challenge was 129 min (16 of 25 vomiting within 120 min; 21 of 25 within 150 min). Thus, a proportionate increase of latency as with copper sulfate by perphenazine would give an average latency of 168 min; this is well within the 5 hr observation period for enterotoxin fed animals.

The results with chlorpromazine were erratic. In the great majority of experiments 2 mg/kg of drug was used. No protection was evident against the S-6 enterotoxin (14/23 of controls *vs.* 13/23 of chlorpromazine treated). Some antiemetic activity against 196E was obtained (27/50 of controls *vs.* 22/50 of drug treated), but most of the protective evidence was obtained with 2 sets of animals. Seven of 9 monkeys responding to 320 mg $CuSO_4 \cdot 5H_2O$ vomited when fed the same level of copper sulfate 90 min after intramuscular injection of 2 mg/kg of chlorpromazine. Latency was increased from 29 to 54 min.

Cyclizine (10 mg/kg) did not protect monkeys against enterotoxin. One experiment with each enterotoxin preparation showed 5/11 vomiting in control groups and 6/12 in drug treated groups.

The highly purified S-6 enterotoxin preparation provoked emesis in 10/10 dogs at 1 μg/kg body weight and in 8/14 at 0.5 μg/kg following intravenous injection. The initial vomiting usually occurred about 2 hrs after injection. Intragastric administration of 25 times the threshold intravenous dose did not cause vomiting. Intravenous chlorpromazine (2 mg/kg) given to dogs challenged with a threshold dose of enterotoxin (1 μg/kg) resulted in 58% protection (5/12). Intramuscular perphenazine (0.1 mg/kg) gave 50% protection (6/12) in dogs challenged with twice the minimal emetic dose. The chlorpromazine and perphenazine dosages used give 31 and 27% protection respectively, in dogs receiving threshold doses of intragastric copper sulfate(7). Thus, perphenazine seems to confer some protection to dogs challenged with enterotoxin; the chlorpromazine results are less suggestive. The limited supply of purified enterotoxin did not allow sufficient work for statistical analysis.

Discussion. Significant reduction of vomiting incidence in monkeys challenged with staphylococcal enterotoxin is obtained when reserpine or perphenazine is used as an antiemetic. Perphenazine is the more potent, but reserpine may be more effective than indicated by the data. Since many control animals fed for the first time vomited rapidly and repeatedly, these animals probably did not receive as much immunizing stimulus as did the drug treated animals. Thus, in the next feeding, sensitivities of animal groups to enterotoxin would be weighted against obtained good protective results. If previously unused animals had been used for all experiments, reserpine might have given better protection.

The number of emetic doses of enterotoxin against which reserpine or perphenazine are

effective was not established, but the nature of the feeding experiments and the results with dogs would suggest that the effectiveness of perphenazine would be limited to only a few times the threshold emetic doses.

At the dosages used, degree of tranquilization of monkeys was not correlated with antiemetic potency. Chlorpromazine produced the greatest tranquilizing effect yet gave only doubtful protection against the 196E enterotoxin, while perphenazine which resulted in least sedation, was effective against both enterotoxins. Whether the basic mechanisms of perphenazine and reserpine protection are the same, or whether the reserpine effect is specifically related to its ability to deplete the biogenic amines from the body binding sites remains to be investigated.

Whether either of the drugs giving protection in these experiments has clinical use remains to be determined. The delayed pharmacological effects of reserpine probably make it unsuitable, but perphenazine was effective even when administered 45 min after enterotoxin. Use of perphenazine in staphylococcal food poisoning outbreaks seems limited, since the majority of cases develop within a short period and recovery begins spontaneously after a few hours of acute symptoms. However, perphenazine may be of value in controlling vomiting caused by staphylococcal enterotoxin in pseudomembranous enterocolitis(13).

The basis of the antiemetic effect in dogs of drugs such as chlorpromazine and perphenazine against the chemoreceptor trigger zone (CTZ) acting emetics is not clear. It has been suggested that at dosage levels which only moderately depress the medullary emetic center the antiemetic activity may be the result of the competition of the antiemetic and the emetic for the receptors in the trigger zone (3,6). On the basis of this interpretation, the results reported here suggest the possibility that staphylococcal enterotoxin may be a CTZ acting emetic.

But monkeys are refractory to emetics such as Hydergine (equimixture of 3 dihydrogenated ergot alkaloids) known to act on CTZ of dogs and cats. This would indicate that CTZ for emesis in the monkey is non-functional from the standpoint of drug action(14). On the other hand, vomiting incidence increases significantly when subemetic dose of dihydroergotamine (related to Hydergine) is administered to monkeys challenged with enterotoxin(10). Moreover, the area postrema of monkeys, which is presumably the CTZ, plays some role in vomiting; its ablation results in elimination of early vomiting response which normally follows 1200 r x-irradiation (14,15).

The above consideration suggests the need for determining the sensitivity of monkeys to enterotoxin following bilateral destruction of the area postrema. Preliminary experiments indicate that the area postrema of monkeys is an important site for emetic action of staphylococcal enterotoxin (to be published).

Summary. Perphenazine and reserpine have significant antiemetic activity against staphylococcal enterotoxin induced emesis in monkeys; chlorpromazine and cyclizine lactate have little or no protective activity. Perphenazine gave the best protection and was effective at 50 μg/kg, intravenously; it inhibited vomiting even when administered 45 min after the enterotoxin had been fed. Pretreatment of monkeys was necessary for protection with reserpine. The effective drug levels did not greatly depress the medullary vomiting center. Perphenazine had some protective effect in dogs being challenged with intravenous injection of enterotoxin.

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A-Norprogesterone, an Androgen Antagonist. (25448)

LEONARD J. LERNER, ALBERT BIANCHI AND ALECK BORMAN
(Introduced by O. Wintersteiner)
Squibb Institute for Medical Research, New Brunswick, N.J.

Typical growth responses of accessory sex organs of the rat or the chick comb to androgen have been inhibited by several substances. These include progesterone(1,2), estrone(2, 3), estradiol(2,4), dehydroepiandrosterone (5), methylcholanthrene(6), 2-acetyl-7-oxo-1, 2,3,4,4a,4b,5,6,7,9,10,10a - dodecahydrophenanthrene(7,8), and 20-methyl-Δ^5-pregnene-3β,20-diol(9); all being steroids or steroid-like in configuration. Structural resemblance of such compounds to natural steroids could form a basis for hormonal antagonism. Recently a series of A-norsteroids was synthesized by Weisenborn and Applegate(10). We studied these steroidal homologs for hormonal and anti-hormonal activities. This report is concerned with A-norprogesterone, which possesses anti-androgenic properties.

Material and methods. Androgenic and anti-androgenic activities. Twenty-three day old male rats (Sprague Dawley strain) weighing approximately 50-60 g were castrated and divided into groups of 4 or 6 animals. Test compounds were dissolved or suspended in sesame oil. A-norprogesterone alone and in combination with testosterone propionate was administered subcutaneously once daily for 7 days starting on day of castration. Animals were sacrificed on day following last injection, and the ventral prostate, seminal vesicles, coagulating gland, levator ani muscle, adrenals, thymus and thyroids were removed and weighed. Intact immature male rats weighing approximately 100 g were subjected to experimental technics similar to those described for the castrated animal. In addition the pituitary, testis, spleen, kidney, heart and liver were removed and weighed. Two day old White Leghorn cockerels, in groups of 24, were injected subcutaneously with a single .5 mg dose of testosterone enanthate (Delatestryl) in sesame oil as in the method described by Dorfman(8). A-norprogesterone was dissolved in sesame oil in several concentrations, and .05 ml was applied on the comb once daily for 7 days starting on day of androgen treatment. The chicks were sacrificed on day following last inunction and the comb and body weights were recorded. *Uterotrophic and anti-estrogenic activities.* Immature female mice of the Swiss Albino strain weighing approximately 7 g were divided into groups of 5 animals. A-norprogesterone dissolved in sesame oil was administered subcutaneously once daily for 3 days, either alone or in combination with a daily dose of .033 μg of estradiol benzoate. Animals were sacrificed on day following last injection. The quantity of uterine luminal fluid was estimated. This fluid was then expressed and the uteri were weighed. *Progestational and anti-progestational activity.* Immature female rabbits in groups of 4 animals were employed in a modification of the McPhail method(11). The animals were given 3 intramuscular injections of 5 μg of estradiol benzoate on days 1, 3, and 5. From sixth day on, they received 5 daily intramuscular injections of progesterone, A-norprogesterone or both compounds. Animals were sacrificed on day following last injection

TABLE I. Anti-androgenic Activity of A-norprogesterone in Immature Castrate Male Rats Treated for 7 Days.

Treatment & daily dose	No. animals	Initial body wt	Final body wt	Ventral prostate	Seminal vesicles & coagulating gland	Levator ani	Adrenals	Thymus	Thyroid
		g				mg			
Sesame oil	42	58.2 ± .5*	95.4 ± .7	11.3 ± .3	11.2 ± .3	21.6 ± .7	31.9 ± .6	410.2 ± 8.7	10.0 ± .3
Testosterone propionate (T.P.), 25 μg	42	61.8 ± 1.7	97.6 ± 1.1	58.1 ± 1.8	52.7 ± 1.7	31.2 ± .9	31.3 ± .6	362.7 ± 12.2	10.5 ± .3
A-norprogesterone (25 mg) + T.P. (25 μg)	3	57.0 ± .6	80.3 ± 4.3	13.1 ± 2.3	13.1 ± .5	17.3 ± 1.6	26.9 ± 1.6	272.7 ± 24.8	8.7 ± .7
A-norprogesterone (5 mg) + T.P. (25 μg)	4	57.5 ± 2.2	97.5 ± 3.9	31.9 ± 1.7	24.6 ± 1.2	20.3 ± 1.3	28.4 ± 1.3	352.6 ± 17.2	9.1 ± .5
A-norprogesterone (1 mg) + T.P. (25 μg)	6	53.2 ± 1.5	97.5 ± 4.0	36.2 ± 1.7	38.6 ± 3.6	29.0 ± 2.0	31.7 ± 1.5	340.2 ± 52.4	9.5 ± .4
A-norprogesterone (5 mg)	4	53.0 ± 1.3	89.5 ± 2.5	12.6 ± 1.5	8.6 ± .2	18.9 ± 1.4	29.3 ± .5	292.5 ± 48.4	9.1 ± .5
" (1 ")	6	55.5 ± 3.9	98.0 ± 2.9	11.6 ± .8	11.1 ± .7	22.3 ± 2.3	32.0 ± 1.3	383.7 ± 44.7	9.4 ± .2

* Staud. error of mean.

and the uteri weighed and graded histologically for glandular development.

Results. The anti-androgenic activity of A-norprogesterone in the androgen treated castrated immature rat is summarized in Table I. The androgen-induced hypertrophy of the ventral prostate was reduced with 1, 5 and 25 mg daily doses of A-norprogesterone by 47, 56 and 96%, respectively. Similarly these doses reduced the hypertrophy of seminal vesicles and coagulating gland by 34, 68 and 95%, respectively. Daily doses of less than 1 mg produced little or no significant changes in accessory sex organ weights of testosterone propionate treated animals. Levator ani weights were decreased to non-androgen treated control levels with 5 mg of this compound. The 25 mg dose of this A-norsteroid slightly reduced body weight gain and levator ani, adrenal, thyroid and thymus weights of the androgen-treated castrate rat. A-norprogesterone in daily doses of 5 mg did not alter ventral prostate weights in non-androgen treated castrates. It did, however, slightly reduce thymus, adrenal, levator ani and seminal vesicle and coagulating gland weights. Preliminary data have indicated that in this assay the oral activity of this substance is of a lower order than is the parenteral activity.

Subcutaneous administration of 25 mg of A-norprogesterone daily for 7 days to 100 g intact male rats resulted in 26% decrease in ventral prostate weight, a 46% decrease in seminal vesicle weight but no significant reduction in coagulating gland or levator ani weights (Table II). The adrenal and thyroid weights were slightly decreased. The thymus, pituitary, testis, spleen, heart, kidney and liver weights were unchanged.

A - norprogesterone antagonized comb growth effect of injected testosterone enanthate as well as comb growth of non-androgen-treated cockerels (Fig. 1). A single injection of the androgen increased the absolute comb weight by 100%, and the comb to body weight ratio by a similar percentage. This comb growth stimulation was completely prevented by total dose of 5 mg of A-norprogesterone. Doses of .04, .2 and 1.0 mg of this

TABLE II. Effect of 7-Day Treatment with A-norprogesterone in Intact Male Rats.

Treatment	Daily dose, mg	Initial body wt (g)	Final body wt (g)	Ventral prostate (mg)	Seminal vesicles (mg)	Coagulating gland (mg)
Sesame oil control		101.0 ± .6*	138.3 ± 2.7	62.8 ± 2.6	34.6 ± .5	10.0 ± .6
A-norprogesterone	25	97.5 ± .9	130.8 ± 1.7	46.2 ± 9.7	18.6 ± 3.0	9.0 ± .9

Treatment	Daily dose, mg	Levator ani (mg)	Adrenals (mg)	Thymus (mg)	Thyroid (mg)
Sesame oil control		32.8 ± 3.5	35.4 ± 1.2	370.7 ± 27.1	12.7 ±.4
A-norprogesterone	25	30.4 ± 2.1	26.3 ± .7	363.9 ± 2.4	9.5 ±.6

* Stand. error of mean.

compound suppressed androgen stimulated comb growth by 24, 41 and 78%, respectively. When administered to non-androgen treated birds, comb weight was reduced by 18%. Body weight gain was unaltered by any of the treatments.

Uterine weights of immature female mice were not increased after administration of 1 mg total doses of A-norprogesterone. This level of compound was unable to alter significantly the uterine hypertrophy induced by estradiol benzoate. A-norprogesterone, therefore, is not estrogenic or anti-estrogenic at this dose.

Intramuscular administration of a total dose of 5 mg of A-norprogesterone failed to produce any progestational effects in estrogen primed immature rabbits. The same dose of this A-norsteroid did not antagonize the progestational activity of 1 mg of progesterone in these primed animals.

Discussion. Results of studies in rat and chick indicate that A-norprogesterone possesses anti-androgenic properties. This activity is not limited to inhibition of exogenous androgen but also manifests itself against endogenous androgen, since in non-androgen treated animals the compound induces a decrease in weight of rat accessory sex organs and of chick combs. It has been reported that reduction in ventral prostate and seminal vesicle weights can be obtained by pituitary gonadotrophin inhibition such as that elicited by estrogen administration (12). On the other hand our results in castrate rats treated with androgen show that inhibition of accessory sex organ hypertrophy by this A-norsteroid is not dependent upon an anti-gonadotrophin effect. A-norprogesterone, at dose levels employed, was devoid of androgenic, estrogenic, anti-estrogenic, progestational and anti-progestational activities. The androgen antagonism, therefore, does not appear to relate to known hormonal effects. The decrease or absence of end organ growth response cannot be readily attributed to any general toxic effect of the compound, since the inhibition was seen at dose levels that caused no significant changes in body weights. It would appear, therefore, that A-norprogesterone competes with androgen at the end organ sites.

Summary. A-norprogesterone antagonizes testosterone induced accessory sex organ hypertrophy in the immature castrate male rat and comb growth stimulation in the chick. Growth of the ventral prostate, seminal vesicles, adrenals and thyroids of intact rats and

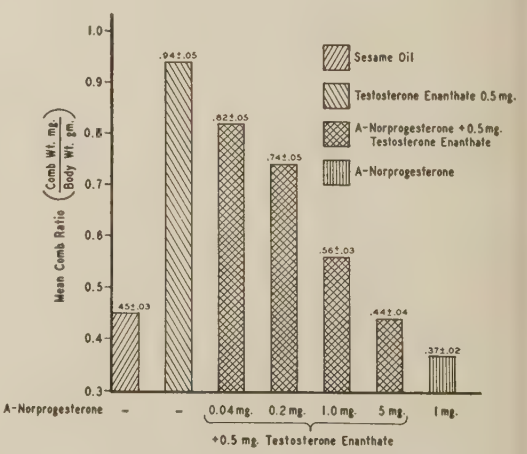

FIG. 1. Anti-androgenic activity as measured by inhibition of androgen-stimulated chick comb growth. Each bar represents the mean comb ratio ± stand. error of the mean. Initial body weights, 36.8 g to 38.7 g; final body weights, 81.7 g to 85.2 g (24 chicks/group).

the chick comb in animals not treated with androgen were significnatly reduced by administration of this compound. A-norprogesterone is not androgenic, estrogenic, anti-estrogenic, progestational or anti-progestational under the experimental conditions described.

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Biological Activities of Some 6-Methylated Progesterones.* (25449)

RICHARD L. ELTON, RICHARD A. EDGREN AND DAVID W. CALHOUN
Division of Biological Research, G. D. Searle & Co., Chicago, Ill.

Biological studies with a growing list of synthetic steroids have revealed marked increases in progestational potency that accompanied introduction of a 6α-methyl group into the molecule(1,2,3,4,5,6). Recent reports have indicated that adrenal depression is encountered in rats treated with some of these agents(7,8) and an earlier report from this laboratory showed that a relationship existed between progestational and estrogen-antagonistic potencies of relatives of 19-nortestosterone(9). The present report compares the progestational, estrogen - antagonistic and adrenal depressant properties of a series of 6-methylated derivatives of progesterone.

Materials and methods. Progestational activity was determined using our modification of uterine carbonic anhydrase assay(6) and the intrauterine assay of McGinty(10). Where possible, potency estimates for uterine carbonic anhydrase test were obtained from dose-response curves by comparing doses of compounds that produced approximately 50-60 enzyme units/100 mg tissue. Progesterone, administered subcutaneously in daily dose of 0.1 mg, produced this response and was used as the standard of comparison for compounds administered by either buccal or parenteral routes. Antagonism of estrone-induced uterine growth was determined in intact immature mice according to previously described methods(9). Briefly, estrone at 0.3 μg, which produced uterine weights averaging about 35 mg in controls, was mixed with test compounds. Estrogen antagonistic potency was determined from the dose of antagonist estimated to produce a 50% block of growth increment (oil controls averaged about 10 mg). Adrenal depressant activity was determined in spayed female rats. The animals were spayed at 30 days of age and received the test compound for 14 days, beginning 10 days after spaying (8).

Results. The metrotropic effects of various steroids are summarized in Table I. Introduction of 6α-methyl group into progesterone or 17α-acetoxyprogesterone was generally accompanied by increases in potency in each of the metrotropic assays. 6α-Methylprogesterone, which showed a slight decrease in potency in the estrogen antagonism test, was an

* Synthetic steroids used were prepared by Drs. P. B. Sollman, C. G. Bergstrom and W. M. Hoehn of the Division of Chemical Research, G. D. Searle and Co. and Dr. B. Löken, formerly of Root Chemical Inc., Roosevelt, Puerto Rico.

TABLE I. Progestational and Estrogen-Antagonistic Activity of Various 6-Methylated Steroids.

Compound	Progestational potency						Estrone-antagonist assay	
	Uterine carbonic anhydrase				Intrauterine assay			
	Subcut.		Buccal					
	N*	Rel. pot.	N*	Rel. pot.	N*	Rel. pot.	N*	Rel. pot.
Progesterone	96(56)	1	96(56)	1	45(22)	1	13 (4)	1
6α-CH_3-Progesterone	8 (3)	5	4 (2)	.2	12 (6)	5	8 (2)	.7
6-CH_3-6-Dehydroprogesterone	6 (2)	2			3 (2)	.5	14 (3)	.7
17α-OAc-Progesterone	8 (3)	10	5 (2)	.2	5 (3)	1	7 (2)	1.2
6α-CH_3-17-OAc-Progesterone	12 (4)	30	17 (6)	5	14 (6)	25	9 (2)	2.9
6β-CH_3- "	8 (2)	20	7 (3)	2	9 (3)	5	10 (2)	1
6-CH_3-6-Dehydro-17-OAc-Prog.	9 (3)	25	8 (2)	17	8 (2)	10	5 (1)	.6
6α-CH_3-21-F-17-OAc-Prog.	17 (4)	40-50	14 (5)	17	23 (7)	50	4 (1)	6.3
6-CH_3-6-Dehydro-21-F-17-OAc-Prog.	20 (6)	40	10 (3)	20	18 (5)	50	3 (1)	2.4

* Under N the parenthetical number indicates No. of tests in which compound was evaluated; number in front of parenthesis indicates total No. of groups of animals employed (4 rabbits/group; 8 to 10 mice/group).

exception. Fluorine substitution at C-21 further augmented activity of 6α-methyl-17-acetoxyprogesterone; this material was 40-50 times more potent than progesterone on subcutaneous administration, 17 times buccally, 50 times on intrautreine application and 6.3 times more potent than the standard in the estrogen antagonism test.

One compound was available in which the methyl group at carbon 6 had the β-configuration, *i.e.*, 6β-methyl-17α-acetoxyprogesterone. In each assay this compound appeared to be less potent than its 6α-methylated isomere, although it was more active than its parent compound.

Three compounds were tested in which the configuration of the 6-methyl group was planar: 6-methyl-6-dehydroprogesterone, 6-methyl-6-dehydro-17-acetoxyprogesterone and 6-methyl-6-dehydro - 17-acetoxy-21-fluoroprogesterone. In general the potencies of these agents were the same as or slightly reduced from those of corresponding 6α-methyl isomers. A notable exception to this was 6-methyl-6-dehydro-17-acetoxyprogesterone which had an oral potency 3 times that of the 6α-methyl isomere; on parenteral administration its potency was decreased in the intrauterine and estrogen antagonism tests.

Discussion. On the basis of these studies there seems to be a general correlation between results of parenteral and buccal administration of these materials in the carbonic anhydrase test. However, 3 materials in the 17-acetoxyprogesterone series, 6-methyl-6-dehydro, the 21 fuoro-6-methyl, and 6-methyl-6-dehydro-21-fluoro-derivatives are much more potent buccally than one would predict from their parenteral potencies. The intrauterine test appeared to be qualitatively correlated with uterine carbonic anhydrase assay. As we pointed out previously, estrogen antagonistic and progestational activities show some relationship(9). A similar correlation is suggested in these studies, although one material, 6α - methyl - 17α - acetoxy-21-fluoroprogesterone, was more potent as an antagonist than would have been predicted on the basis of its potency in the carbonic anhydrase test.

A number of compounds caused atrophy of the adrenal glands in treated rats. In certain experiments progesterone has shown this activity to a limited extent, although replicate experiments have not been consistent. In this study progesterone and 17-acetoxyprogesterone were inactive in this respect (Table II). All 6α-methylated derivatives of 17-acetoxyprogesterone tested were active. Introduction of a methyl group in the α position of carbon 6 in the progesterone molecule resulted in adrenal depressant effects, but this compound did not appear to be more potent than the 6α-methyl derivatives of 17α-acetoxyprogesterone. 6-Methyl - 6 - dehydroprogesterone appeared to be inactive; it seems strange that introduction of the double bond in this com-

TABLE II. Adrenal Depressant Activity of 6-Methylated Steroids in Rats.

Compound	Test	Dose (mg)	N	Adrenal wt (mg)
Oil	1	—	8	50.
	2	—	6	59.1
Progesterone	1	1	8	53.8
6α-CH_3-Progesterone	2	1	5	33.4*
6-CH_3-6-Dehydroprogesterone	2	1	6	53.5
17-Acetoxyprogesterone	1	1	8	57.4
6α-CH_3-17-OAc-Progesterone	1	.3	9	34.8*
	1	1	8	23.2*
6-CH_3-6-Dehydro-17-OAc-prog.	1	1	8	23.2*
6-CH_3-6-Dehydro-17-OAc-21-fluoroprog.	1	1	9	20.6*

* Mean differs significantly from mean of simultaneous oil-treated controls ($P < .01$; Wilcoxon Rank Sum Test).

pound reduced activity while having no effect on activity of the 17-acetoxyprogesterone form. Since several active progestins lack adrenal depressant activity it appears that the 2 activities are not directly related.

Summary. Progestational, estrogen-antagonistic and adrenal depressant activities of a series of 6-methylated steroids was determined. Generally, introduction of a 6α-methyl group increased potency over the parent compound; 6β-methylation of 17-acetoxyprogesterone increased potency to a lesser extent. When the configuration of the 6-methyl group was neither α nor β, potencies generally were about the same as or slightly less than the 6α-methylated form. Exception to this was 6 - methyl - 6-dehydro-17-acetoxyprogesterone which had high oral potency. Adrenal depression was noted following 6-methylation.

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Serum Levels After Single Oral Doses of 6-(α-Phenoxypropionamido) Penicillanate and Penicillin V.* (25450)

CLARKE G. MCCARTHY, HANS A. HIRSCH AND MAXWELL FINLAND
Thorndike Memorial Laboratory, Second and Fourth (Harvard) Medical Services, Boston City Hospital and Dept. of Medicine, Harvard Medical School, Mass.

Isolation of 6-aminopenicillanic acid by Bachelor *et al.*(1) has made possible the preparation of new penicillins and related compounds, not easily accessible in other ways. One such new synthetic penicillin is 6 - (α-phenoxypropionamido) penicillanate.†

* Aided by grant from Nat. Insts. Health.

† Also designated α-phenoxyethyl penicillin (Syncillin, Maxipen); both the powder and the tablets consisted of a mixture of the d- and l- isomers.

The present paper deals with controlled comparison of levels of antibacterial activity resulting in serum from equivalent oral doses of this compound and of phenoxymethylpenicillin (penicillin V).

Materials and methods. The synthetic penicillin, which will be referred to in the Tables as S, was supplied as potassium salt by Bristol Labs as P152, both as sterile powder and as tablets, each containing 268 mg. Potassium phenoxymethyl penicillin (penicillin V) was supplied as powder and as tablets containing 250 mg of penicillin V by Eli Lilly & Co. Each tablet contained the equivalent of 144 mg of 6-aminopenicillanic acid. The 10 subjects of this study were healthy young men weighing 64-95 (av. 75) kg. Each was given, in rotation, 2 tablets of each of these forms of penicillin before or immediately after breakfast with interval of 3 or 4 days between doses. Because of an error in coding, all of subjects repeated the first 2 rotations so that each subject received 6 doses, of which 2 were duplicates; the 2 forms were thus given before or after breakfast twice to each of 5 subjects. Venous blood was obtained before, and again 1, 2, 4, 8 and 12 hours after each dose; those given the dose on a fasting stomach took breakfast after the 2-hour blood. Serum was separated as soon as possible and each specimen was stored in 2 tubes, kept frozen at –20°C until assayed. All specimens in each patient were assayed simultaneously by the 2-fold dilution method in beef heart infusion broth (pH 7.2), using both *Streptococcus 98* and *Staphylococcus aureus 209P*.‡ Sensitivity of these strains to 3 forms of penicillin is shown in Table I. Equal parts of a 10^{-4} dilution of an 18 hr broth culture were added to serum dilutions and incubated at 37°C for 24 hours. Results of these tests are expressed as maximum number of 2-fold dilutions of serums which completely inhibited the test organism. Total activity resulting from each dose was estimated from the area under the curve of penicillin levels and expressed as dilution hours. All second tubes of serum were delivered under code and in the frozen state to the Bristol Labs, where they were assayed by the cup-plate, agar-diffusion method using *Sarcina lutea* (ATCC 9341) as assay organism(2). Results of these tests were compared with standards of both penicillin V and of P152 and expressed as μg/ml of each agent, and total activity from each dose was estimated from the "area" under the blood level curve and expressed as μg-hrs.

‡ These assays were carried out by Clare Wilcox and Joan H. Yarrows.

TABLE I. Sensitivity of Assay Organisms to 3 Forms of Penicillin.

Penicillin	Minimum inhibiting conc., μg/ml* Strep. 98	Staph. 209P
Penicillin G	.008	.031
" V	.013	.026
P152	.031	.063

* Determined by 2-fold dilution method in broth. Each value is avg of 7 determinations done on different days; results were all either identical or varied within a single 2-fold dilution. The minimum concentration detectable by the *S. lutea* cup-plate method was 0.02 μg/ml of both penicillin V and P152.

Results of all assays (excluding those of the second of the same doses given each subject) are summarized in Table II. All control serums obtained before the doses and all serums drawn 8 and 12 hours after the doses yielded no detectable levels. Average levels obtained by any of the 3 methods with each form of penicillin given after breakfast did not differ significantly from those obtained when the same tablets were given on a fasting stomach. Likewise average peak levels obtained from nearly all doses were quite similar, the only statistically significant differences being in the antistaphylococcal and antistreptococcal activity derived from penicillin V taken fasting, which were higher than those obtained from P152 taken after breakfast. Total antistreptococcal and antistaphylococcal activity produced in serum by penicillin V after a meal was also significantly greater statistically than that of the synthetic form given either before or after breakfast, and antistaphylococcal activity of serum resulting from penicillin V after the meal was significantly greater than that obtained from P152 after the meal. The 4-hr levels after the fasting dose of P152 were significantly lower than at the same interval after penicillin V given either fasting or after

TABLE II. Serum Levels after Oral Doses of Penicillin V and 6-(α-Phenoxypropionamido) Penicillanate in Relation to Food.

A. Antistreptococcal and antistaphylococcal activity

Assay organism	Dose*	Avg No. of 2-fold inhibiting dilutions of serum‡ 1 hr	2 hr	4 hr	Peak	Avg total 8 hr activity,† dilution-hr
Strep. 98	Vb	8.1	6.8	3.9	8.2	30.4
	Sb	7.7	6.5	2.8	7.7	25.9
	Va	7.5	6.9	4.6	7.7	32.3
	Sa	7.3	6.3	3.7	7.5	27.7
Staph. 209P	Vb	6.9	5.5	2.3	7.1	21.9
	Sb	6.8	5.1	.4	6.8	15.7
	Va	6.6	5.6	3.1	6.8	24.3
	Sa	6.4	5.2	1.7	6.4	19.2

Significant differences

Strep. 98	Peaks	Vb-Sa ($P < .05$)	Staph. 209P	Peaks	Vb-Sa ($P < .02$)
	Total activity	Va-Sb ($P < .01$)		Total activity	Vb-Sb ($P < .02$)
		Va-Sa ($P < .05$)			Va-Sb ($P < .01$)
					Va-Sa ($P < .05$)
	4 hr level	Vb-Sb ($P < .05$)		4 hr level	Vb-Sb ($P < .02$)
		Va-Sb ($P < .01$)			Va-Sb ($P < .01$)

B. Serum levels based on penicillin standards in cup-plate diffusion method

Penicillin standard*	Dose*	μg/ml of serum (avg)‡ 1 hr	2 hr	4 hr	Peak	Avg total 8 hr activity,† μg-hr
V	Vb	3.0	1.0	.2	3.1	5.1
	Sb	2.8	1.1	.1	2.8	4.6
	Va	2.6	1.3	.3	2.7	5.2
	Sa	2.3	1.1	.2	2.4	3.9
S	Vb	3.2	1.2	.2	3.3	5.4
	Sb	3.0	1.2	.1	3.0	5.1
	Va	2.9	1.3	.2	2.8	5.1
	Sa	2.4	1.1	.2	2.5	4.7

None of differences are significant.

* V = penicillin V; S = 6-(α-phenoxypropionamido) penicillanate; dose was 500 mg and 536 mg, respectively, given orally as the potassium salt; b = dose given on fasting stomach, and breakfast taken after 2 hr blood; a = dose given immediately after breakfast.

† Estimated for 8 hr as "area" under curve of serum levels.

‡ Each value is avg for same 10 subjects; all control seras taken before the doses and all those obtained 8 hr after the doses yielded no detectable levels.

breakfast. No differences in levels obtained by the cup-plate diffusion method, however, achieved statistical significance.

The validity of the comparisons is attested by results of duplicate tests done after the subjects had taken the same tablets on different occasions. Average results of these duplicate tests, each done in 5 subjects, are remarkably similar, and the same was true of assays of the corresponding individual serums.

Comment. These crossover studies indicate that the new synthetic penicillin and penicillin V are absorbed in a remarkably similar manner and degree and that these forms of penicillin yield quite comparable serum levels when given in the same manner to the same individuals at different times. The differences noted, although some of them were statistically significant, are not likely to be of clinical importance.

Other observations not detailed here have indicated some differences in susceptibility of a number of strains of staphylococci to these 2 forms of penicillin and to penicillin G, but these differences, too, are not large enough to be of clinical importance, and, for the most part, are of the same order of magnitude as those shown in Table I.

Synthetic penicillin is also inactivated by penicillinase. In our tests 194 strains of penicillinase-producing staphylococci and 35 which did not produce penicillinase were

streaked on the surface of agar inseminated with *Sarcina lutea* and containing P152 in excess of minimum inhibiting concentration of the latter, in a modified Gots test for penicillinase(3). Colonies of Sarcina developed around the surface growth of all the penicillinase producing strains of *Staphylococcus aureus* in these plates in the same manner as in similar plates in which penicillins V and G were used, indicating that all 3 of these penicillins are at least qualitatively similar in their susceptibility to penicillinase activity.

Conclusions. Potassium 6-(α-phenoxypropionamido) penicillinate and potassium phenoxymethyl penicillin are absorbed in essentially the same manner in normal men and produce comparable levels of antibacterial activity in the serum. Both of these penicillins are qualitatively similar to penicillin G in their susceptibility to penicillinase produced by *Staphylococcus aureus.*

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B_{12} Metabolism and Multiple Sclerosis.* (25451)

John S. O'Connor, Robert L. Davis,† Orthello R. Langworthy and Bacon F. Chow

Dept. of Biochemistry, School of Hygiene & Public Health, Johns Hopkins University, and Depts. of Neurology and Psychiatry, Johns Hopkins Hospital, Baltimore, Md.

Previous reports(1-5) indicate that the metabolic pattern as well as urinary and serum levels of Vit. B_{12} may differ according to the disease state in question, such as pernicious anemia(6-8), liver disease(9-11), myelogenous leukemia(12-13), diabetes mellitus (14-15) and others. Since Vit. B_{12} has been implicated in metabolism of proteins, carbohydrates, fats and nucleic acids, it is not too surprising that alterations in its disposition by the body in diverse disease states have been observed. Vit. B_{12} has been also implicated with multiple sclerosis (MS) because of the beneficial effect of Vit. B_{12} in preventing and even reversing the demyelinizing effects of pernicious anemia. For this reason, treatment of MS with large doses of Vit. B_{12} has been investigated in many clinics with variable results(16-18). It is, therefore, of interest to ascertain (1) whether there are biochemical signs of Vit. B_{12} deficiency in subjects with MS by measuring Vit. B_{12} serum levels and glutathione (GSH) contents in the erythrocytes and (2) to determine whether such patients can handle orally or parenterally administered Vit. B_{12} as the clinically healthy subjects.

Materials and methods. The Vit. B_{12} containing Cobalt^{60} had a specific activity of approximately 800 μc/mg for urinary excretion studies. Preparation of "resting" cells of *Lactobacillus leichmannii* ATCC 4799 has been described(9). *Serum.* Whole blood was obtained by venipuncture from fasting patients. Serum obtained by centrifugation was stored in frozen state until assayed. Serum Vit. B_{12} levels were determined according to the method of Skeggs, *et al.*(20). *Vit. B_{12} absorption test.* To estimate absorption of orally administered Vit. B_{12}, the Schilling procedure(6) was modified. The details have been published elsewhere(19). *Glutathione in erythrocytes.* Determination of GSH in red blood cells was carried out in duplicate aliquots of 0.5 ml of blood cell suspension by the nitroprusside test as modified by Grunert and Phillips(21-22). *Urinary excretion test.* To determine amount of injected Vit. B_{12} re-

* The authors appreciate the Cobalt^{60} labeled vit. B_{12} kindly supplied by Dr. Charles Rosenblum, Merck & Co., Rahway, N. J.

† Present address: Veterans Admin. Center, Bay Pines, Fla.

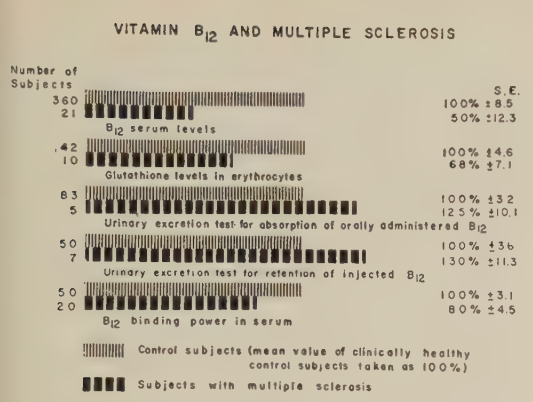

FIG. 1.

tained by tissues, the procedure of Lang, *et al.* (23) was used. All patients here described were diagnosed by several neurologists and followed up in a University setting. These diagnoses were established for years and no recent cases were included. Except for 2 or 3, all were ambulatory patients and none were acutely ill hospitalized patients. All showed progressive disease but no acute exacerbation.

Results. Fig. 1 summarizes results of Vit. B_{12} serum levels, urinary excretion test for both absorption of orally administered and retention of intramuscularly administered Vit. B_{12} serum Vit. B_{12} binding capacity, and finally GSH contents of erythrocytes. In presenting our results, the mean value of clinically healthy control subjects was assigned a level of 100% and results obtained from studies on MS patients were compared percentage-wise to this level.

Serum Vit. B_{12} concentrations as obtained by microbiological assay technic employing *L. leichmannii* ATCC 4797, demonstrated that the MS group had a significantly lowered mean value (50% of normal group). The range of these serum Vit. B_{12} contents was 220 $\mu\mu$g/ml to <50 $\mu\mu$g/ml. Ten of 21 cases had levels well within our range for pernicious anemia patients (50 $\mu\mu$g/ml), whereas some were within normal range.

Since severe Vit. B_{12} deficiency with such low Vit. B_{12} serum levels is often accompanied by marked diminution in the GSH content of red blood cell(22), the latter level was likewise quantitated. The observed data suggest that the MS group has a diminished GSH content of erythrocytes (68% of normal group). This method indicates concentration of sulfhydryl groups, and since GSH is responsible for almost all of the SH of the red blood cell, the measurement is essentially specific for this compound.

To determine whether Vit. B_{12} deficiency is due to poor absorption of orally administered Vit. B_{12}, the urinary excretion test was performed. In the third pair of bars, comparisons are made of urinary excretion results for absorption of orally administered Vit. B_{12} between MS patients and clinically healthy control subjects. The procedure employed followed the directions of Schilling(6) for estimation of orally absorbed Vit. B_{12}. The data indicate that there was greater radioactivity in urine of MS patients than in urine of the control group. However, when ability to retain injected $Co^{60}B_{12}$ was measured, it was observed that the MS group excreted considerably more of injected Vit. B_{12} as quantitated by urinary excretion of this vitamin. The next 2 sets of bars summarize this finding. The last set of bars summarizes data which indicate that serum of MS patients bind somewhat less Vit. B_{12} than the control group (100% vs. 80%). The procedure, following microbial absorption technic, quantitates residual binding capacity of serum for Vit. B_{12}.

In view of data indicating increased excretion of Vit. B_{12} by MS subjects when administered Vit. B_{12} intramuscularly, studies were initiated to compare excretion patterns of MS patients upon administration of Vit. B_{12} in aqueous solution or as a repository. To this end, 5 MS patients (Group A) were treated with Vit. B_{12} (1000 μg), whereas 8 MS patients (Group B) were treated with Depinar (500 μg). Group A excreted 600 $\pm$ 80 μg of Vit. B_{12} in 24 hrs, whereas Group B only 25.1 $\pm$ 2.5 μg in similar period. When such patients (Groups A) were administered Vit. B_{12} at 500 μg level, approximately one-half of 1000 μg excretion level was observed. It is readily observed that the MS patients receiving "Depinar" excreted a fraction of Vit. B_{12} activity of the group receiving Vit. B_{12} (cyanocobalamin) (Table I).

TABLE I. Urinary Excretion of MS Patients Administered Vit. B_{12} and "Depinar" Intramuscularly.

Group	Treatment	No.	Total B_{12} activity in 24 hr urine (μg)
A	Vit. B_{12} (1000 μg)	5	660 $\pm$ 80 *
B	"Depinar" (500 μg)	8	25.1 $\pm$ 2.5

* Stand. error of mean.

Discussion. Vit. B_{12} has been associated with MS since Booth and co-workers attempted to treat this disease with massive doses of Vit. B_{12}. Levin(17) and Leveboullet(18) have likewise explored Vit. B_{12} as an aid in management of MS. Results have been variable and difficult to evaluate because of spontaneous remissions characteristic of this disease. Vit. B_{12} has great efficacy in preventing and even reversing the demyelinizing effect of pernicious anemia. It is perhaps this dramatic therapeutic property of Vit. B_{12} that has led to investigations in metabolism of this vitamin in MS. Our study demonstrates possible biochemical signs of Vit. B_{12} deficiency as indicated by low Vit. B_{12} serum levels. It is also evident that MS patients as a group have diminished GSH contents of the red blood cell. Ling and Chow(22) found decreased level of non-protein sulphydryl groups in erythrocytes of animals fed diets deficient in Vit. B_{12}. Patients with pernicious anemia had marked diminutions of soluble sulphydryl compounds in blood, which increased upon administration of Vit. B_{12}. The changes observed in blood sulphydryl levels were primarily due to those in concentration of GSH. Since these patients were not administered Vit. B_{12}, and many of their serum B_{12} levels indicated deficiencies, further work will determine whether Vit. B_{12} corrects this diminution in sulphydryl compounds in blood. Decreases in blood GSH may also occur in certain conditions other than pernicious anemia, such as potassium or sodium deficiency(24), diabetes, and some chronic infections. Whether the decreased GSH level observed in the MS group is due to a derangement in productive capacity of the soluble sulphydryl compounds or to an increased destruction due to oxidation or conjugation with metabolites produced during the course of MS is not known. The deficiency is not due to poor absorption of orally administered Vit. B_{12}, but to poor tissue retention so that absorbed Vit. B_{12} is readily excreted and, therefore, unavailable for metabolic use. Since parenterally administered cyanocobalamin in an aqueous solution is rapidly excreted in urine even by non-multiple sclerotic subjects, search was made for a preparation containing Vit. B_{12}, with repository effects. It was found that intramuscular administration of Vit. B_{12} in the form of Vit. B_{12}-tannate derivative, "Depinar," resulted in marked retention of this vitamin as measured by urinary excretion. Additional studies(25) on both control human adults and rats demonstrated that "Depinar" administered intramuscularly resulted in marked elevation of serum Vit. B_{12} levels which persisted over several weeks. It was likewise observed that rats administered "Depinar" tagged with Co^{60} Vit. B_{12} had elevated radioactivity in the organs. These results indicated that when Vit. B_{12} was provided in depot form, it would be excreted at a slower rate and thus provide a continuous source of Vit. B_{12} for metabolic functions.

Future studies should indicate whether Vit. B_{12} supplied in the form of "Depinar" will increase low blood levels of B_{12} in several MS patients as observed in a normal group for prolonged periods of time.

Summary. Data have been presented to suggest that MS patients as a group do not metabolize Vit. B_{12} and GSH, quite in the same quantitative manner as a control group. Their average Vit. B_{12} serum level is lower, as is their ability to retain the vitamin when administered intramuscularly, although they absorb adequately. Average GSH content of the red blood cell was reduced in the MS group. When Depinar, a Vit. B_{12}-tannate derivative, was administered to such patients, Vit. B_{12} was excreted much more slowly in urine than aqueous Vit. B_{12}.

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Effect of Inhibitors of Oxidative Phosphorylation on Biosynthesis of Cholesterol and Precursors by Liver Homogenates.* (25452)

LEMUEL D. WRIGHT AND MARCIA LOEB
Graduate School of Nutrition, Cornell University, Ithaca, N. Y.

Essentially whole homogenates of rat liver readily convert mevalonic acid (MVA) into non-saponifiable material (NSF) and cholesterol(1). This synthetic capacity is ordinarily retained during a control preincubation period but preincubation with RNAse abolishes capacity to convert MVA to NSF(2). RNAse-treated homogenates, in contrast to controls, are devoid of ATP but homogenates pretreated with RNAse and heated liver extract from which ATP has been removed by anion exchange chromatography maintain a level of ATP and convert MVA to NSF(3,4, 5). In the liver homogenate system studied the amount of added MVA incorporated into NSF appears to be directly proportional to the level of ATP existing at the time of MVA addition. Whole homogenates of liver convert MVA to NSF under aerobic but not under anaerobic conditions(6). Biosynthesis of NSF occurs under anaerobic conditions, however, if the homogenate is first centrifuged at intermediate speed to remove "tissue fragments" relatively rich in ATPase activity. The foregoing observations emphasize the significance of systems concerned with the generation of ATP in the biosynthesis of NSF and cholesterol. ATP is known to be specifically required in the phosphorylation of MVA, the phosphorylation of MVA-5-phosphate and the concerted decarboxylation and dehydration of MVA-5-pyrophosphate(7). Oxidative phosphorylation is the primary source of ATP in aerobic homogenates of liver. Accordingly some inhibitors of oxidative phosphorylation have been studied for the extent to which they indirectly inhibit biosynthesis of NSF and cholesterol and the data

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obtained are reported in this paper.

Materials and methods. The biosynthetic experiments involved preincubation of 5 ml aliquots of 200 x g supernatant fraction of rat liver homogenate prepared as previously described with 5 ml amounts of buffer or inhibitor in buffer for a period of 30 min in stoppered 125 ml Erlenmeyer flasks(1-6). Each flask also contained 1 mg ATP and 1 mg DPN and each flask was aerated with a stream of oxygen prior to preincubation. Following preincubation each flask was opened and 1 ml MVA-2-C^{14} solution added, the contents were aerated and the flasks then reincubated for an additional 3 hrs. After the final incubation the homogenates were saponified, extracted with petroleum ether, the extracts dried with sodium sulfate, filtered, evaporated, taken up in scintillation mixture and counted. Previous studies have shown(1) that under the experimental conditions employed in these studies the counts found in the NSF fraction are essentially cholesterol or other digitonin-precipitable material. In some of the experiments sodium succinate was added to the homogenates at a level of 0.05 M. Supplementation with this substrate has been found to reduce incidence of experiments where no incorporation of MVA occurs even in control flasks.

Results. The data obtained are summarized in Table I. By far the most active inhibitor is 2,4-dinitrophenol. Biosynthesis of NSF from MVA is essentially nil at 10^{-5} to 10^{-4} M concentrations of the inhibitor. Methylene blue and acriflavin are quite active with essentially maximum inhibition at 10^{-4} to 10^{-3} M. At sub-inhibitory levels methylene blue appears to stimulate the system. Potassium cyanide is completely inhibitory at 10^{-2} M but shows no inhibition at 10^{-3} M. Sodium fluoride is inhibitory in the range of 10^{-3} to 10^{-2} M. Calcium chloride is not inhibitory at 10^{-3} M and higher concentrations were not studied. Sodium azide is only partially inhibitory at 10^{-2} M.

Discussion. The results obtained show that homogenates of rat liver preincubated with classical inhibitors of oxidative phosphorylation no longer incorporate MVA into NSF. The mechanism seems obvious that in the presence of the inhibitors ATP which is required in a number of the metabolic conversions of MVA to NSF is either not formed or is more rapidly hydrolyzed.

A number of the compounds studied were less active, presumably as inhibitors of oxidative phosphorylation, in the present system than in other systems that have been studied involving isolated liver mitochondria. Since the present system is a very crude one it is to be expected that specific components against which the inhibitors act are present in large excess and consequently require more inhibitor for inactivation.

TABLE I. Summary of Inhibition Data.

Exp. #	Inhibitor	Conc., M	cpm	% inhibition
1	None		3483	
	Potassium cyanide	1×10^{-3}	3592	0
	2,4-Dinitrophenol	"	12	100
	Sodium azide	"	3698	0
	Calcium chloride	"	3228	7
	Methylene blue	"	74	98
2	None		3546	
	2,4-Dinitrophenol	1×10^{-3}	3	100
	"	1×10^{-4}	10	100
	Methylene blue	"	20	100
	Sodium fluoride	1×10^{-1}	30	99
	Idem	1×10^{-2}	1496	58
3*	None		4095	
	Methylene blue	1×10^{-3}	17	100
	Idem	1×10^{-4}	4539	0
	"	1×10^{-5}	5239	0
4*	None		3731	
	Methylene blue	1×10^{-3}	0	100
	Idem	1×10^{-4}	6538	0
	Acriflavin	1×10^{-3}	0	100
	"	1×10^{-4}	4035	0
	"	1×10^{-5}	3751	0
5	None		4308	
	Sodium fluoride	1×10^{-1}	339	92
	Idem	5×10^{-2}	544	87
	"	1×10^{-2}	1735	60
	"	5×10^{-3}	1547	64
	"	1×10^{-3}	3559	17
	"	1×10^{-4}	3769	13
6	None		5329	
	Sodium fluoride	1×10^{-1}	53	99
	Idem	1×10^{-3}	4459	16
7	None		5549	
	2,4-Dinitrophenol	1×10^{-4}	251	96
	"	1×10^{-5}	5111	8
8	None		1513	
	2,4-Dinitrophenol	1×10^{-4}	6	100
	"	1×10^{-5}	19	99
	Potassium cyanide	1×10^{-2}	16	99
	Sodium azide	"	793	48

* Signifies experiments carried out in the presence of 0.05 M sodium succinate.

Although some of the compounds studied are quite active as indirect inhibitors of NSF and cholesterol biosynthesis there is little possibility that an inhibitor of oxidative phosphorylation would have utility in practical control of cholesterol biosynthesis. Oxidative phosphorylation is of such fundamental significance in the metabolism of most cells in an aerobic environment that no basis of selective action against cholesterol biosynthesis in particular would appear to exist.

Summary. Classical inhibitors of oxidative phosphorylation particularly 2,4-dinitrophenol inhibit the biosynthesis of non-saponifiable material and cholesterol from mevalonic acid by rat liver homogenates. Presumably in the presence of inhibitors a level of ATP is not maintained for the phosphorylations and the concerted decarboxylation and dehydration essential in utilization of mevalonic acid.

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Utilization of Pyridoxine N-Oxide by Rats.* (25453)

TAKETAMI SAKURAGI AND FRED A. KUMMEROW
Dept. of Food Technology, University of Illinois, Urbana

When pyridoxine was treated with a mixture of glacial acetic acid and hydrogen peroxide, addition of one atom of oxygen resulted (1). Based on analogous reactions observed with a variety of pyridine derivatives(2), the product was believed to be pyridoxine N-oxide(1). The currently available data indicate that some biological systems are capable of reducing a heterocyclic compound containing N-oxide functions(3,4). In addition, the biological formation of N-oxides as a normal constituent has been reported(4,5). By means of bioautography and microbiological assay, pyridoxine N-oxide retained some Vit. B_6 activity for growth of *Saccharomyces carlsbergensis*, suggesting that yeast could reduce a portion of the N-oxide(1). In the present study, the metabolism of pyridoxine N-oxide by rats has been investigated.

Methods. Male rats 3 weeks of age were placed on basal diet† for 10 days and then evenly divided into 5 groups of 6 rats each with respect to body weights. During assay period, the basal ration and water were fed *ad lib.* In addition, rats were supplemented once each week for 4 weeks with distilled water, or distilled water which contained 20 μg of pyridoxine hydrochloride, 100 μg of pyridoxine hydrochloride, or equivalent amounts of pyridoxine N-oxide hydrochloride. *Paper chromatography and bio-autography.* Paper chromatography was conducted on Whatman No. 1 filter paper at room temperature in a descending system. The solvent was the upper layer of a mixture of isoamyl alcohol, pyridine and water (2:1:2, *v/v*). The spots of Vit. B_6 components as well as pyridoxine N-oxide were detected by spraying the papergram with

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† Each 10 lbs. of basal ration free from both fats and vit. B_6 contained 7.8 lb glucose, 1.8 lb vitamin-free casein, 0.4 lb Wesson salts(6), 9 g choline chloride, 4.5 g inositol, 10 mg thiamine chloride hydrochloride, 15 mg riboflavin, 100 mg nicotinic acid, 90 mg D-calcium pantothenate, 45 mg menadione, 0.5 mg biotin, 15,000 I. U. vit. A, 0.15 mg calciferol and 30 mg alpha-tocopheryl acetate.

TABLE I. Average Body Weight Gains of Male Rats Supplemented with Pyridoxine or Pyridoxine N-oxide over a Period of 4 Weeks.

Supplement*	Levels of supplement,† μg/rat/wk		
	0	20	100
	g		
Pyridoxine	6.2 ± 4.9‡(5)§	37.3 ± 4.1 (6)	83.7 ± 6.7 (6)
" N-oxide		32.3 ± 4.4 (6)	79.7 ± 6.2 (6)

* Supplementation was conducted once a week at beginning of every week by oral administration of aqueous solution of vitamin preparations.

† Vitamin level is indicated as pyridoxine hydrochloride.

‡ Stand. dev. calculated according to the formula: $\sqrt{\Sigma d^2/(n-1)}$.

§ Figures in parentheses indicate No. of rats used in each group.

N,2,6-trichloro-*p*-quinoneimine dissolved in benzene followed by exposure to ammonia vapor, or ferric chloride dissolved in ethanol. For bioautography, the papergram which had been dried was cut into 21 sections so that each section represented 0.05 of an R*f* unit. These sections were numbered from 0 to 20 as the R*f* values increased, and individually placed in a tube containing 9 ml of assay medium(7). After 1 hr at room temperature, the paper sections were removed and the tubes steamed in autoclave at 100°C for 10 min. After cooling, each tube was inoculated with 1 ml of a suspension of a 24 hr culture of *S. carlsbergensis* (A.T.C.C. 4228). Incubation was conducted at 30°C for 18 hr and growth of yeast in each tube was measured, using Coleman No. 9 Nepho-Colorimeter, degree of growth expressed in terms of optical density against distilled water at 655 mμ. *Excretion of Vit. B_6 from rats after administration of pyridoxine and pyridoxine N-oxide.* Six normal male rats weighing between 395 and 410 g were divided into 3 groups of 2 rats each. Two ml of distilled water, 2 ml of distilled water containing 5 mg of pyridoxine hydrochloride, or 2 ml of distilled water containing equivalent amount of pyridoxine N-oxide hydrochloride was administered *via* stomach tube. Urine was collected for 15 hr; urine samples were pooled within each group and diluted to 100 ml. To each paperstrip, 0.01 ml of urine thus prepared was applied and developed for bioautography. One-tenth ml of urine specimen after supplementation with pyridoxine N-oxide was also mixed with 0.1 ml of concentrated hydrochloric acid. Five mg of sodium nitrite was added to the acidic solution and the mixture heated on steam bath for 10 min., 0.02 ml of final solution was used for bio-autograph.

Results of growth assay with rats using pyridoxine or pyridoxine N-oxide as sole source of Vit. B_6 indicated that pyridoxine N-oxide retained Vit. B_6 activity (Table I). Differences between body weight gains which resulted upon supplementation with pyridoxine and pyridoxine N-oxide were statistically insignificant at the 5% level.

Efficient utilization of pyridoxine N-oxide as source of Vit. B_6 by intact rats was further supported by data of urine samples collected after administration of pyridoxine hydrochloride or pyridoxine N-oxide hydrochloride. Supplementation with pyridoxine and its oxide apparently gave similar bioautographic patterns (Fig. 1: *B, C*). When the urine specimen, obtained after oral administration of pyridoxine N-oxide, was treated with nitrous acid before bioautography, the low R*f* peak disappeared, while the high R*f* peak remained (Fig. 1; *D*). This confirms that the former peak represented pyridoxamine. Under the experimental conditions, peaks of pyridoxal and pyridoxine tended to fuse, and thus the high *Rf* peak as observed represented pyridoxal and/or pyridoxine. Although the N-oxide was far less active than pyridoxine in supporting growth of assay organism,‡ the appearance of a large, high R*f* peak after administration of the N-oxide suggested that excretion of pyridoxine N-oxide as such in urine, if any, was negligible (Fig. 1; *C*). The patterns as presented were obtained using 0.01 ml

‡ Apparent activity of pyridoxine N-oxide in supporting growth of assay organism, *S. carlsbergensis*, varied from time to time, usually ranging 5-17% of that of pyridoxine on a molar basis.

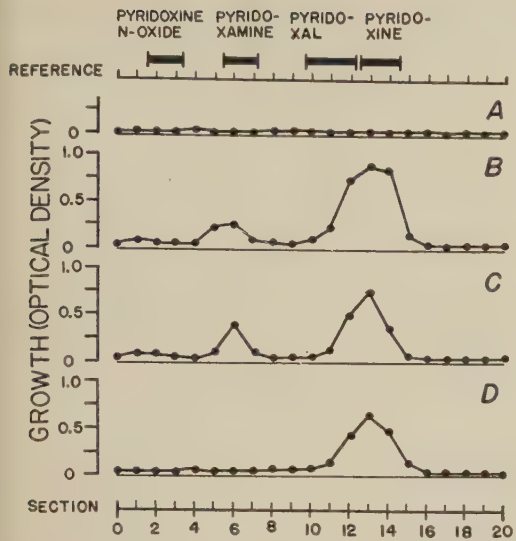

FIG. 1. Bioautographic pattern of vit. B_6 in rat urine. Urine samples were collected over a period of 15 hr after oral administration of *(A)* none, *(B)* pyridoxine • HCl, or *(C)* pyridoxine N-oxide • HCl. The pattern *D* represented urine collected after administration of pyridoxine N-oxide and subsequently treated with nitrous acid. The low *Rf* peak represents pyridoxamine and the high *Rf* peak represents pyridoxal and/or pyridoxine.

each of urine specimens. Even 0.05 ml of urine specimen from rats which had been supplemented with pyridoxine N-oxide failed to reveal a peak corresponding to pyridoxine N-oxide.

Since it became evident that reduction of pyridoxine N-oxide to Vit. B_6 readily occurred in intact animals, its possible transformation *in vitro* was investigated. Fifty μg of pyridoxine N-oxide hydrochloride was incubated with 0.5 ml of a 0.1 M phosphate buffer (pH 7.4) for 4 hr at 37°C in an open tube in the presence of 50-55 mg of fresh rat liver slice (approximately 1.5 mm thick) and of other additives. The liver slices were prepared from 45-55 day old normal male rats weighing 180-230 g. After incubation, degree of reduction was calculated from the increase in apparent Vit. B_6 activity in the digest, as measured directly with *S. carlsbergensis*(8). Reference tubes were concurrently prepared with pyridoxine hydrochloride as well as pyridoxine N-oxide hydrochloride. The calculation was based upon the assumption that Vit. B_6, presumably pyridoxine, which had been formed from pyridoxine N-oxide did not undergo further changes. Under conditions employed, 50 μg of pyridoxine hydrochloride, which had been incubated in place of pyridoxine N-oxide hydrochloride, retained its full activity when assayed with *S. carlsbergensis*.

Liver slices alone were unable to reduce pyridoxine N-oxide. In the presence of liver slice plus 800 μg of DPNH§ or 5 mg of a preparation of alcohol dehydrogenase, reduction of pyridoxine N-oxide was noted. In both cases, however, only approximately 5% of N-oxide originally added was reduced. When the alcohol dehydrogenase was used as an additive, further addition of DPN and/or ethyl alcohol, or addition of Mg^{++} and/or Mn^{++} failed to enhance the reduction. It was also noted that reduction of pyridoxine N-oxide by rat liver slices did not occur in the presence of one or more of the following additives: ATP, DPN, TPN, TPNH, glucose 6-phosphate and glucose 6-phosphate dehydrogenase. Under the variety of conditions tested, whenever no increase in Vit. B_6 activity after incubation was observed, Vit. B_6 activity attributable to the original pyridoxine N-oxide was fully recovered, suggesting that the N-oxide remained unchanged.

A variety of Vit. B_6 derivatives retaining vitamin activity for growth of rats are known (7,9-11). Except for the ω-methyl analogs of Vit. B_6, they are believed to regenerate free Vit. B_6 *in vivo*(7,11). Our results seemed to indicate that *in vitro* pyridoxine N-oxide *per se* could not replace the biochemical functions of Vit. B_6, which, however, is readily formed *in vivo* from the N-oxide. It has been reported that the N-oxide of a metabolite sometimes serves as an antimetabolite(12). Pyridoxine N-oxide, however, does not seem to act as an antivitamin B_6 in rats as well as in yeast.

Summary. The evidence shows that intact rats are capable of reducing pyridoxine N-oxide to Vit. B_6 and utilizing it as a source of vitamin. Pyridoxine N-oxide supported growth of rats almost as efficiently as did an equivalent amount of pyridoxine.

§ Preparations of DPNH (Lot No. 79-741), yeast alcohol dehydrogenase (Lot No. 79-700), DPN, TPN, and TPNH were purchased from Sigma Chemical Co., St. Louis, Mo.

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Serologic Comparison of Mammary Tumor Viruses from 3 Strains of Mice.* (25454)

PHYLLIS B. BLAIR (Introduced by Howard A. Bern)

Dept. of Zoology and Its Cancer Research Genetics Lab., University of California, Berkeley

The antigenic nature of the mouse mammary tumor virus is recognized. Sera of rabbits, rats, and guinea pigs(1-3) previously injected with extracts of virus-containing tissues are capable of neutralizing the virus in tumor extracts, both *in vivo* and *in vitro*. Normal sera of these animals, on the other hand, have little or no effect on the virus. The present experiment was designed to investigate possible antigenic differences between mammary tumor viruses possessed by different strains of mice. To eliminate any possible effect arising from tissue antigen differences, one strain (BALB/cCrgl) served as donor for all viruses. A comparison was made between the antigenicity of the viruses carried by 2 different strains of mice (C3H/Crgl and A/Crgl) and also between the viruses carried in a strain (A/Crgl) and a subline of it (A/Crgl/3, formerly designated A/vi), which are known to differ in effect upon tumor development(4). In addition, possible tissue differences were investigated by a comparison of one virus in 2 genetically different hosts (A/Crgl virus in A/Crgl and in BALB/cCrgl mice). The effectiveness of rabbit antisera in neutralizing the activity of the viruses was measured by tumor incidence in susceptible virus-free test mice injected with virus and antiserum.

Materials and methods. Antigen preparation: Mammary tissue was taken from the following groups of mice (Table I): BALB/c females (virus-free C); BALB/c females fostered on C3H (C3H virus in C); BALB/c females fostered on A/3 (A/3 virus in C); BALB/c females fostered on A (A virus in C); and A females (A virus in A). The donor females were permitted to breed, and were sacrificed during the third week of their second lactation. Mammary gland pairs #2, 3, and 4 were dissected out. The mammary glands from 2 females in a group were homogenized in a Waring blendor with 24 ml sterile saline for a total of 2 min. The mixture was spun in an International Clinical Centrifuge for 30 min at 1540 g. The pellets were discarded and the supernatants were then spun for 90 min in the multispeed attachment of an International Model U Centrifuge (maximum 30,000 g). The supernatants were discarded, and the pellets were resuspended in saline (.5 ml/g of original tissue weight for injection into rabbits, 2 ml/g of original tissue weight for injection into mice). The solu-

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TABLE I. Mammary Tumor Development in BALB/c Females Given Various Injections of Mammary Tumor Virus and Rabbit Antiserum.

Antigen source for antiserum	Virus	No. of mice	No. with tumors	% tumors	Mean tumor age (mo)
No serum	A virus in C	24	10	42	12.6
	A/3 *idem*	23	14	61	8.2
	C3H "	24	13	54	10.7
	A virus in A	22	7	32	11.6
Normal rabbit serum	A virus in C	23	12	52	11.9
	A/3 *idem*	25	13	52	8.5
	C3H "	16	6	38	11.3
	A virus in A	20	9	45	12.3
Virus-free C	A virus in C	19	11	58	11.0
	A/3 *idem*	20	16	80	8.3
	C3H "	19	8	42	10.4
	A virus in A	16	7	44	13.6
A virus in C	A virus in C	30	1	3	16.5
	A/3 *idem*	16	0		
	C3H "	26	0		
	A virus in A	28	0		
A/3 virus in C	A virus in C	25	0		
	A/3 *idem*	15	0		
C3H virus in C	A virus in C	27	1	4	8.5
	C3H *idem*	27	0		
A virus in A	A virus in C	27	1	4	14.0
	A virus in A	26	0		

tion was cleared by spinning in the International Clinical Centrifuge for 15 min and was placed in the refrigerator overnight. The material was kept under refrigeration during the preparation procedure. *Antiserum preparation*: Six-month-old male albino rabbits were injected intramuscularly once weekly for 4 weeks with the antigen preparation derived from 1 g of lactating mammary tissue. Four days after last injection, blood was withdrawn from heart, allowed to clot overnight, and centrifuged to separate serum from the clot. At the same time, blood was collected from 2 rabbits which had not received any injections. *Neutralization*: Equal amounts of rabbit serum and saline were combined in test tubes with the virus preparation from 1 g of lactating mammary tissue in each 20 ml of final solution. They stood at room temperature for 2 hours. Each mouse received .2 ml liquid intraperitoneally. Therefore, the total amount of virus preparation given to each test mouse was that derived from .01 g of lactating mammary tissue. The test animals were 17 to 24-day-old BALB/c females, weaned at 5 weeks and bred for 2 litters. The offspring were discarded within 24 hours after birth. All tumorous females were sacrificed. Histologic sections were made of tumors taken from approximately ¾ of tumor-bearing animals. The experiment was terminated when tumor-free animals were 18 mos. old.

Results are summarized in Table I. Test mice which received virus alone, or virus combined with normal rabbit serum, or virus combined with serum from rabbits previously injected with virus-free BALB/c tissue, developed mammary tumors. Thus the injected dose of virus was sufficient to cause tumor development, and the viruses are not inhibited by normal rabbit serum nor by antisera against virus-free tissue.

However, in experiments where the virus preparation was mixed with rabbit antiserum against a mammary tumor virus, tumor development did not occur (with 3 isolated exceptions). Neutralization occurred regardless of whether the antiserum had been prepared against the same or another virus, either a virus from a different strain of mice, or a virus from the same strain (different subline) but with a different effect upon tumor development.

Bittner and his co-workers(3,5,6) using A, C3H, and (A x C3H) F_1 mice have reported that the tissues of the donor mouse may have

a reversible effect upon ability of antisera to neutralize the virus. In the present experiment the source of the virus had no effect upon efficiency of the neutralization: antisera against A virus neutralized the A virus from both BALB/c mice and A mice, regardless of whether the virus preparations used in antisera production had been derived from A mice or BALB/c mice.

In control groups a significantly earlier age of tumor development was found in the group of mice given A/3 virus, than in groups given A (in BALB/c or in A tissue) or C3H virus. This difference with regard to the A/3 and the A viruses will be discussed elsewhere.

Summary. No antigenic differences were found when the mouse mammary tumor viruses from 3 different strains or sublines (one in 2 different host strains) were compared, using the technic of *in vitro* virus neutralization with rabbit antisera followed by injection into susceptible virus-free test mice. Normal rabbit serum and antiserum against virus-free tissue did not neutralize the virus. Antiserum against any of the viruses neutralized that virus and all others tested. Neutralization of the virus was not affected by the tissue source of the virus.

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Experimental Preparation Showing Effect of Reserpine on Tissue Resistance.* (25455)

A. S. LEONARD, W. O. GRIFFEN, JR. AND OWEN H. WANGENSTEEN
Dept. of Surgery, University of Minnesota Medical School, Minneapolis

Reserpine has been shown to be a potent agent in abetting ulcer diathesis both clinically and in the laboratory(1,2,3). The mechanism of acute ulcer production by reserpine is not clearly understood, although it has been shown that intravenous injection of reserpine causes a marked increase in HCl secretion in human patients(2) and also augments Heidenhain pouch secretion in the dog(3). Whether reserpine has any effect on tissue resistance, however, has not been clearly assessed. Susceptibility of cat's esophagus to the erosive action of human gastric juice has been well documented in this laboratory(4). Using a modification of this technic, the peripheral effect of reserpine has been observed and is here reported.

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Method. Adult mongrel cats were used. The animals were divided into 2 groups. In both groups, the animals were anesthetized with pentobarbital, 30 mg/kg, and positive pressure respiration was maintained automatically through a cervical tracheostomy. In Group I the cervical esophagus and gastric cardia were cannulated for outflow tracts. A left thoracotomy through 7th interspace was achieved and the esophagus mobilized. A No. 26 T-tube with ¾ cm "limbs" was inserted through longitudinal incision in mid esophagus, each limb being secured in place by an umbilical tape. By alternate occlusion of the limbs of the T-tube, the upper or lower segments of the esophagus could be perfused separately. In Group II animals the entire esophagus was utilized for perfusion, with the perfusate being introduced through a cannula in the cervical esophagus and drained at 20 cm outflow pressure at the lower end of the

esophagus(5). The perfusate used throughout this experiment was human gastric juice collected on ice in the postoperative period from 11 p.m. to 7 a.m. from patients who had not undergone gastric surgery. The specimens were analyzd first for pH, free acid and pepsin and were then acidulated to a pH of 1.6 with 0.1 N HCl, providing an optimal range for peptic activity. The technic of perfusion in Group I cats was as follows: An overnight specimen was divided into 2 equal portions of at least 250 cc each. The control segment of the esophagus was perfused for 2 hours at a rate of 30 drops per minute with a constant outflow pressure of 20 cm. At the end of this time, the perfused portion of the esophagus was thoroughly irrigated with saline. Reserpine, 0.5 mg, was given *via* the femoral vein, which had been exposed previously, and after a 2 hour period, the alternate segment of the sophagus was perfused for a period similar to the control by changing the position of the occluding clamp. The control and test portions were alternated to preclude the possibility of increased susceptibility of the cephalad or caudad segment of the esophagus. In the Group II animals, the overnight specimens were also split into portions of at least 250 cc each. The control cat was perfused for a 2 hour period with this gastric juice, and the test cat was perfused for a similar 2 hour period, after a 2 hour period to permit reaction of 0.5 mg of reserpine given intravenously through the femoral vein. At the conclusion of the perfusions, the animals were sacrificed and the esophagi were removed. The control and test sides were graded 0 to 5+ with reference to the esophageal injury noted(5); 0, representing no injury, 1+, representing slight mucosal reddening, and 5+, denoting perforation of the esophagus.

TABLE I. Comparison of Digestion of Cat's Esophagus between Control and Reserpine Treated Animals.

Grade of digestion	Group I (12 cats)		Group II (7 pairs)	
	Control	Reserpine treated	Control	Reserpine treated
0	5	0	5	1
1+	5	2	0	1
2+	1	3	1	1
3+	1	5	1	2
4+	0	0	0	0
5+	0	2	0	2

1+ signifies mucosal reddening without injury; 5+ denotes perforation.

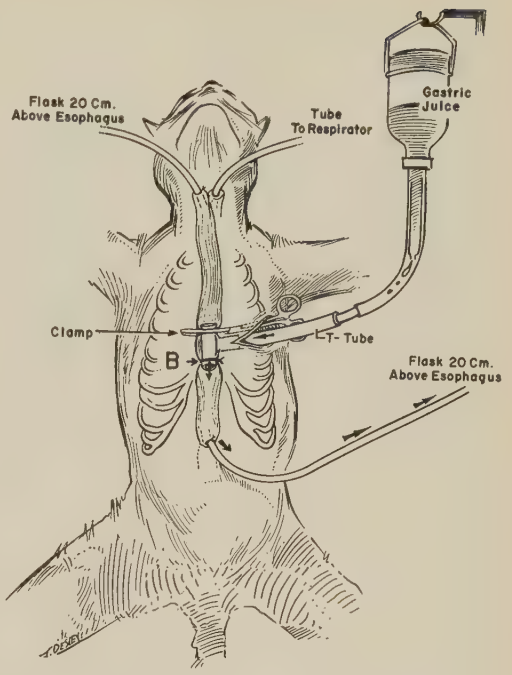

FIG. 1. Tissue resistance preparation using the same cat's esophagus as the control and test specimen.

Results. There were no perforations in the control animals of either Group I or Group II, whereas in the reserpine treated experiments there were 4 perforations (Table I). In Group I, 7 of the 12 segments of esophagi treated with reserpine demonstrated an injury of 3+ or greater as opposed to only 1 of 12 in the control specimens, and indeed, 10 of 12 specimens, or 83.3%, showed a significant increase of digestion of 2+ or more after reserpine administration. In the Group II animals, 4 of 7 cats treated with reserpine demonstrated an esophageal injury of 3+ or more as opposed to only 1 out of 7 of the control animals. In fact, 5 of the 7 or 71.4% of reserpine tested preparations demonstrated 2+ or higher difference in digestion over the control group.

Discussion. The results attending intravenous administration of reserpine indicate a significant decrease in tissue resistance of cat's esophagus perfused with human gastric juice.

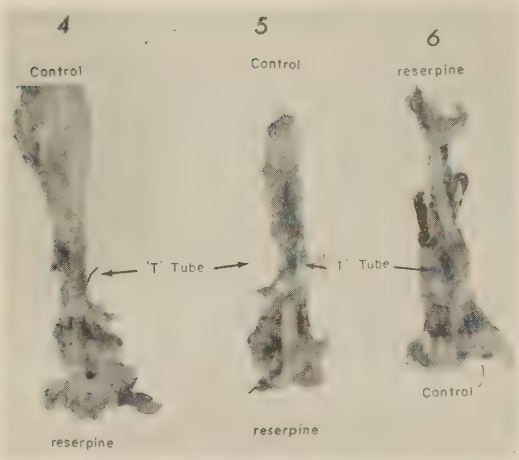

FIG. 2. Perfused preparations representing Group I or divided cat esophagus. Specimen 4: control side grade 0 digestion; reserpine treated side grade 1+. Specimen 5: control side grade 1+ digestion; reserpine treated side grade 3+. Specimen 6: control side grade 2+ digestion; reserpine treated side grade 5+.

This is revealed in increased digestion observed in the divided esophageal perfusion as well as in experiments using a separate cat's esophagus.

The mechanism this decreased tissue resistance produced by reserpine remains obscure. Increased ACTH release through hypothalamic stimulation(6) with resultant corticosteroid secretion may play a role in this increased tissue susceptibility. Serotonin release(7) and local factors such as vascular spasm or decreased blood flow because of hypotension may be important. These various possibilities are being investigated by use of this preparation.

Summary and conclusion. The I.V. administration of reserpine lowers significantly the susceptibility of the intact cat's esophagus to injury by digestive action of human gastric juice.

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Familial Primary Amenorrhea Due to Testicular Feminization: A Human Gene Affecting Sex Differentiation.*† (25456)

THEODORE T. PUCK, ARTHUR ROBINSON AND J. H. TJIO
Dept. of Biophysics, Florence R. Sabin Labs., University of Colorado Medical Center, Denver

The chromosomal constitution of individuals suffering from gonadal dysgenesis (Turner's syndrome) has been demonstrated to consist of only 45 chromosomes, the autosomes of which were normal in number and morphology, but the sex chromosome condition being XO(1,2). Such patients do not undergo ordinary developmental changes of puberty, but remain essentially sexually infantile females. The Barr Test for nuclear chromatin, which when positive indicates existence of at least 2 X chromosomes, is negative. Chromosomal delineation of the somatic cells of these patients demonstrated that in man, the Y chromosome contains genes necessary for development of male characteristics, a situation unlike that of *Drosophila,* but similar to that in the mouse(3). Another patient with clinical diagnosis of female pseudohermaphroditism was studied(2) and reported to display cytologically normal female (XX) chromosomes. Hungerford, *et al.* reported a case of true hermaphroditism(4) to have displayed an XX chromosomal condition. The present report deals with another female patient, exhibiting primary amenorrhea asso-

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† Contribution No. 94.

ciated with testicular feminization (male pseudohermaphroditism). This condition is of especial interest because, while it resembles gonadal dysgenesis in some features, it differs markedly in others, among these being the presence of a strong hereditary component. The genetic basis of a condition clinically like this has been discussed but not resolved(5a, 6a,6b). A detailed clinical report of this 25-year-old woman will be presented elsewhere. The diagnosis appeared unequivocal and was based, among other findings, on female external genitalia, well developed breasts and feminine habitus, primary amenorrhea, negative Barr Test for nuclear chromatin, a normal urinary excretion of follicle stimulating hormone and 17-keto steroids, and a pedigree indicating transmission of the defect only through the maternal line.

Methods. Samples of skin were obtained, and the cells cultivated and examined for chromosomal morphology, by standard methods previously described(7,2). The one modification over previous procedures consisted in sampling an area of healing superficial skin lesion instead of normal skin, a substitution which appears to shorten the time needed to establish a stably growing culture. A piece of skin from the back of the neck or behind the ear is anesthetized with dichloro-tetrafluoro-ethane (Frigiderm, W. T. Brachvogel, Los Angeles, Calif.), then abraded over an area 1 x 0.1 cm, to depth just sufficient to reach the dermis by means of a Dermabrasion instrument (Mill-Bilt Equipment Co., N. Y.). The abraded area is covered with a Bandaid, and allowed to develop a scab. Seventy-two hours after the abrasion, the scab is washed in 70% alcohol, dried, lifted off aseptically with forceps and deposited in Petri dish containing nutrient solution, fortified with tested human cord serum, as previously described (7a). The scab, which may weigh about 5 mg, is cut in about 10 pieces with sterile scissors, the medium becomes cloudy with detached cells. The dish is incubated, with regular medium changes as previously described, in incubator at 37°C, containing 5% CO_2 in air almost saturated with water, with temperature and gas composition carefully regulated. In about 7 days the cells multiplied when they may be trypsinized, and re-seeded into new vessels. The cultures so obtained, like others described by us(7a), grow with a generation time of approximately 20 hours, and maintain stable chromosomal constitution throughout production of virtually unlimited numbers of progeny, so long as the specified conditions are maintained. The healing wound forms a new scab after the first one is removed. This may be re-sampled if necessary. No scar results from this procedure.

Results. Typical chromosomal complements from cells of our patient are shown in Fig. 1. There are 46 members. Ideograms from 10 different mitoses have been prepared by arranging chromosomes in accordance with the standard classification scheme of Tjio and Puck(8). A typical example is shown in Fig. 2. It is evident that the autosomes are normal but the sex chromosomal constitution is XY, rather than female, XX. In examination of more than 100 mitoses, the chromosome number of 46 was uniformly obtained except in 4 cells, 2 of which had 44 and 2 of which had 45 chromosomes, the supposition being that these represented chromosome losses occurring during fixation. The Y chromosome was always clearly identifiable.

In our patient, the only pedigree data so far available consist in written reports supplied by the patient and her mother. Efforts are being made to study other members of the family, who are widely distributed. Against the time when these additional data can be obtained directly, a preliminary summary is furnished here of the familial history, which seems justified, in view of the unusual intelligence and cooperativeness of the patient and her mother, and the ease with which the condition of primary amenorrhea can be recognized. The data indicate that primary amenorrhea has occurred in some females of every generation in 4 consecutive generations. The number of apparent females outnumbers the males by approximately 3 to 1. The condition has been transmitted exclusively in the maternal line, typical of this form of primary amenorrhea(5a). Hence, a defect on the Y chromosome as the causative factor of this condition is ruled out. No known consanguinity has occurred in the family through-

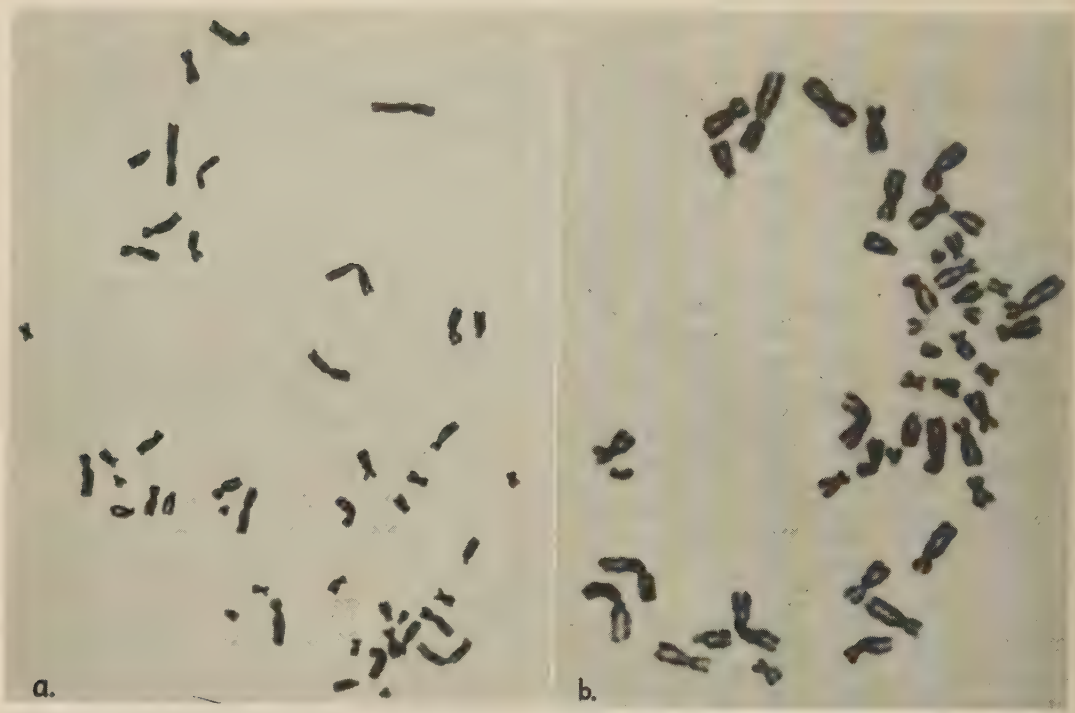

FIG. 1. Typical chromosomal complements of this patient's cells grown in tissue culture as described.

out these 4 generations. Of particular importance is the fact that the patient's mother, maternal grandmother, and two maternal cousins, have all borne children, but did not initiate menses until ages of 19 to 21. This delayed menarche in families of patients like that described here has been rarely recorded, although it is mentioned in one member of a family studied by Pettersson and Bonnier(6).

FIG. 2. Ideogram of chromosomes of the patient here described, showing the typical male chromosomal pattern.

The nature of the primary genetic lesion in cases exhibiting a condition clinically like the present one has been reviewed(5a). The genetic evidence has been carefully weighed and the conclusion reached that these individuals have an XY chromosomal condition with a defective gene, which could be either a sex-linked recessive or a sex-limited autosomal dominant; a smaller alternative probability was judged to exist that the XXY condition or some other chromosomal anomaly is the causative factor(5a).

The chromosomal constitution of this patient rules out the XXY possibility. An XY/46 chromosomal constitution has also been ascribed to a patient of this type by Chu and Giles(5b), and to 4 other patients with presumably the same condition, reported by Lennox(5c). It has also been described by Severinghaus(5d) in a male pseudoherma-

phrodite of somewhat more male type than the present patient. While the detailed chromosomal constitutions and clinical findings in the cases of Lennox and of Chu and Giles have not yet been presented, the concordance of these results as well as the genetic data previously available makes it at least a reasonable presumption that the disease condition here considered is fairly regularly associated with the given chromosomal constitution. Moreover, similarities in findings in all these cases also make it very unlikely that the chromosome here described as a Y is in reality a portion of a fragmented X.

To decide whether the genetic defect is carried on the X chromosome, or whether it is autosomal but is never transmitted through the male line because of its sterilizing influence on male carriers, data are needed from families containing this defect along with known markers, especially sex-linked ones like color blindness or hemophilia as Grumbach and Barr have noted(5a). Evidence with some bearing on this question is available from a recently reported case of hemophilia in a "girl" baby with a 46/XY chromosomal constitution(9). While that child was too young for the decision to be made as to whether her condition was unequivocally classifiable as pseudohermaphroditism with feminizing testes, it seems highly likely, in view of the correspondence obtained between clinical diagnosis and chromosomal constitution in all other studies so far available in such patients. Moreover, the maternal pedigree presented for that child indicates a very high preponderance of females, as would be expected in this condition.

On this assumption, and assuming further the absence of other complicating factors, one can examine the distribution of both these defects in that family: If the sex-differentiation defect were on the X chromosome and closely linked to the hemophilia gene, it could be present either on the same X chromosome as the latter, or on the other X chromosome. In the former case, the sex differentiation defect would virtually always accompany the sex-linked defect in the chromosomal males. In the latter, the 2 defects would not appear in the same XY individual. Thus, it would not be possible to find 2 siblings, both with the XY constitution, both exhibiting the hemophilia defect, but only one of which possessed the sex differentiation defect here considered. But in the pedigree of the patient reported in data of Nillson *et al.*(9), a sexually normal male and the pseudohermaphroditic patient appear in the same set of siblings, both exhibiting the hemophilia condition. Therefore, the conclusion may be drawn, subject to the uncertainties noted, that the sex differentiation gene in this case is autosomal, or else, if sex-linked, is so far distant from the hemophilia gene as to permit crossing over with high probability. It cannot be assumed, however, from the clinical resemblance between 2 independently arisen hereditary conditions, that they have been caused by a mutation at the same locus, no matter in how many details their syndromes agree.

Accumulation of additional pedigrees of cases clinically like our present one, involving at the same time differences in other marker genes, should eventually permit a determination of whether the genetic basis of the condition in question is uniform, and if so, whether it is present on the X chromosome or on a given autosome; or whether the condition represents a genetically heterogeneous class of cases, the mutant genes for which may be at any one of a number of sites, like those causing intersexuality in *Drosophila* and those causing the waltzer or shaker syndrome in mice(10).

In Turner's syndrome the XO condition is present and menses, plus other aspects of normal female development, fail to occur. While the condition of such an XO patient indicated that genes on the Y chromosome are necessary for development of maleness, data from the kind of patients here considered indicate that these are not sufficient for full expression of male character. The gene responsible for the condition of pseudohermaphroditism with feminizing testes in XY individuals like our patient appears to exercise an important influence on development in both sexes: in the heterozygous condition in an XX individual, it appears capable of delaying menarche for about 8 years. When present with an XY chromosomal constitu-

tion, however, it prevents expression of maleness, producing an essentially female condition, but without reproductive function.

After this paper was submitted, Jacobs *et al.*(11) described bone marrow cell analysis from 4 patients with testicular feminization, all of whom had a 46/XY chromosomal constitution. Thus the correlation between XY sex chromosomes in females and the condition of testicular feminization is strengthened. Moreover, in one of these cases, as in the case of hemophilia discussed by us, a color blind male pseudohermaphrodite and a color blind normal male occurred in the same set of siblings. Therefore, since the gene responsible for testicular feminization is not closely linked to *either* of 2 different sex-linked genes, its autosomal location becomes highly probable.

Summary. A female patient with "male pseudohermaphroditism with feminizing testes" had a 46/XY chromosomal constitution, a fact confirming clinical and genetic deductions drawn by previous investigators largely on the basis of pedigree data, and in accord with recent brief reports on the chromosome condition in other such patients. A detailed chromosomal analysis is provided. Some previous cases having similar clinical manifestations had been recognized by previous workers as being caused either by an autosomal dominant or a sex-linked recessive genetic defect. Analysis of pedigrees from similar cases demonstrates the causative gene defect to be not strongly linked to either of 2 sex-linked genes, hemophilia and color blindness. Hence it is probably autosomal. Of particular importance is the finding that in the family of our patient, all female transmitters of the defect for whom data were available exhibited a delayed menarche of approximately 8 years. This situation has been rarely noted previously, and requires study as to its universal accompaniment of this condition. It is evident that in the present case the presence of this gene in heterozygous condition in an XX individual causes a delay of menarche, while in an XY individual it results in suppression of the male characteristics and a partial development of female traits. It is pointed out that more than one gene locus might be able to produce clinical effects like those here described. Study of the action of such genes appears highly important to problems of human development.

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Summation of Protein Anabolic Effects of Testosterone Propionate and Growth Hormone.* (25457)

CHARLES D. KOCHAKIAN†
University of Alabama Medical Center, Birmingham

Simultaneous administration of testosterone propionate and a growth hormone preparation at their respective maximum effective

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TABLE I. Summation of Protein-Anabolic Effects of Growth Hormone (GH) and Testosterone Propionate (TP).

Treatment			Nitrogen retention						Max increase body wt		
			Maximum*			Total					
Rats, No./group	GH, u/day	TP, mg/day	GH	TP	GH + TP	GH	TP	GH + TP	GH	TP	GH + TP
			mg/day			mg			g		
4	1.1	.5	14	60	70	273	1050†	1239†	+ 4	+16	+21
4	1.1	.5	27	59	68	491	869	987	+11	+16	+25
3	2.2	.5	84		122	853		1455	+14		+23

* Avg of second, third and fourth periods (7 days) when nitrogen retention was at maximum.
† Retention was more prolonged.

doses to castrated mice resulted in a summation of their respective effects on body weight and protein deposition(1). These studies have been extended to nitrogen balance studies in adult castrated rats.

Procedure. The rats (Sprague-Dawley) were maintained in individual metal metabolism cages in air-conditioned room at 25.5-26.6° and with artificial light regulated at 12 hr/day. They were fed the previously described diet(2) in an amount that would maintain body weight and constant urinary nitrogen excretion. Some rats had been used in another similar experiment. They were approximately 1.5 years old at beginning of the first of these studies. Body weights were 320-480 g and nitrogen intakes were 291-388 mg/day. The rats were grouped to give a uniform distribution of both body weight and urinary nitrogen excretion. Growth hormone‡ and testosterone propionate§ were injected subcutaneously at the same time each day. The dose of testosterone propionate was known from previous studies(2) to produce a maximal protein anabolic effect. The initial dose (Exps. 1 and 2, Table I) of growth hormone also was expected to give a maximal response but the preparation proved to be less active than the previous material(3). Routine of feeding, urine collection and analysis was the same as previously described(2).

Results. Body weight. The androgen produced the expected maximal response in body weight(2). Simultaneous administration of growth hormone resulted in a summation of the effect of two hormones on body weight. Degree of summation was almost complete in the 2 experiments with the lower dose of growth hormone but was less than the sum at the higher dose level.

Nitrogen metabolism. The growth hormone at both dose levels produced a further increase in nitrogen retention both at maximal level and total amount. Summation of the effects of the 2 hormones was nearly complete in the first and third experiment but was intermediate in the second experiment.

Discussion. Ability of growth hormone to superimpose its protein-anabolic action on the maximal effect of testosterone propionate is in agreement with studies in mice. These data provide further support to the independent protein anabolic action of androgens and growth hormone(3,4). The action of these hormones not only is specific for certain tissues but very likely they stimulate synthesis of protein in the same tissue, *e.g.*, muscle, for different functions. These hormones, thus, function in a supplementary manner in growth and development of the various tissues of the body.

Summary. Simultaneous administration of testosterone propionate and growth hormone to castrated rats resulted in a summation of their respective effects on body weight and nitrogen retention even at maximal doses.

† Experimental work was performed at Oklahoma Med. Research Fn. with technical assistance of Sarah McConnell.

‡ Growth hormone was provided by Parke, Davis and Co.

§ Testosterone propionate was provided as perandren, 10 mg/ml by Ciba Pharmaceutical Products.

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Role of Microbial Fermentation in Improvement of Barley by Water Treatment.*† (25458)

J. M. Thomas, L. S. Jensen, K. C. Leong and J. McGinnis
Washington State University, Pullman

Fry *et al.*(1) showed that the nutritional value of barley for chicks was improved by a simple water treatment. Similar results were obtained by other workers(2,3,4). Since studies on the mechanism(s) involved in improvement were not reported, experiments were conducted to investigate the role of microbial action.

Methods. Microbial counts on samples of ground barley treated in different ways were made to correlate improvement in nutritional value with microbial growth. Two treated samples were taken from the drying oven in sterile 250 ml Erlenmeyer flasks containing glass beads. The flasks were shaken 2 hours to grind large aggregates to fine particles. Serial dilutions were made of weighed samples and bacterial counts determined, using tryptone-glucose extract agar and incubating 41 hours at 37°C. No attempt was made to enumerate fungi. Since Kent-Jones and Amos(5) found *B. subtilis* and *B. mesentericus* common inhabitants of wheat flour, *B. subtilis* (ATCC 9943) and an unidentified, spore-forming, Gram positive rod isolated from our feed preparation area, were used to inoculate barley in 2 experiments. Treatments involving addition of water to grain consisted of adding 1¼ parts liquid (50°C) to 1 part coarsely ground grain, mixing, spreading approximately ½″ deep on trays, and drying in a forced draft oven for 24 hours at 70°C. The treated grain was reground through a ⅛″ screen and mixed in the diets. Wet barley was autoclaved in trays (Tables I and III), or metal 30 lb egg cans (Table II) to facilitate sterilization. Hot sterilized barley in cans was inoculated with culture material, aseptically mixed, sealed, stood 4 hours at room temperature, spread on trays, and placed in drying oven. Two 20-hr nutrient agar cultures were swirled separately for 2 minutes with 50 ml 0.5% peptone water each; both were added to barley as the inoculum. Barley autoclaved in trays was removed and dried immediately in the oven. The large inoculum added directly to dry barley diets was 2 separately grown 200 ml, 36-hr nutrient broth cultures; the small inoculum was one 100 ml culture. Fungal enzyme‡ (fungal amylase) was used as positive control, since our previous work has shown it improves the nutritional value of dry barley diets(6). Day-old New Hampshire Chicks of both sexes were randomly distributed into groups of 10 birds each,

TABLE I. Effect of Various Treatments of Barley on Microbial Count and Chick Growth.

Diet	Avg wt 2 wk (g)	Organism $\times 10^6$/g
Barley	90	2
Sterilized* barley	116	5
Water-treated barley	122	94
Sterilized*(dried), water-treated	122	154
" inoc. fungal amylase (4.4 g/kilo)	126	177

* Autoclaved 10 p.s.i., ½ hr.

TABLE II. Effect of Bacterial Additions to Sterile Barley on Chick Growth and Feed Efficiency.

Barley treatment	Drying temp. (°C)	Avg wt 2 wk (g)§	Feed gain
None		84	2.24
Sterilized,‖ inoculated*‡	95	84	2.13
" ‡‖	70	93	2.05
" ‡‖ " †	70	112	1.74
" ‡‖ " *	70	113	1.78
Fungal amylase (4.4 g/kilo)		108	1.85
Water-treatment	70	110	1.71

* Unidentified organism.
† *B. subtilis* (ATCC-9943).
‡ No standing period.
§ Treatments within a common bracket are not significantly different from each other. Treatments without a common bracket are statistically significant at the ($P < .01$) level.
‖ Autoclaved 15 p.s.i., ¾ hr.

* Scientific Paper No. 1902, Washington Agri. Exp. Stations, Pullman. Project No. 1291.

† Portions of these data presented at 48th Annual Meeting Poultry Sci. Assn., Iowa State University, Ames.

‡ Merck Sharp and Dohme, Rahway, N. J.

TABLE III. Effect of Adding Whole Bacterial Cultures to Dry Barley Diets and Autoclaving Water-Treated Barley on Chick Growth and Feed Efficiency.

Barley treatment or addition	Avg wt 2 wk (g)*	Feed gain
400 ml media	108	2.18
None	109	2.05
100 ml culture	116	1.98
400 " "	125	1.96
Fungal amylase (4.4 g/k), 400 ml culture	130	1.88
Water-treated barley	131	1.70
Fungal amylase (4.4 g/k)	134	1.78
Water-treated (dried), sterilized (125% H_2O),† dried 95°C	134	1.76
Water-treated (dried), sterilized (25% H_2O),† air dried	135	1.79
Water-treated, 400 ml culture	137	1.74

* Treatments within a common bracket are not significantly different from each other. Treatments without a common bracket are statistically significant at the ($P < .05$) level.

† Autoclaved 15 p.s.i., ½ hr. Dry water-treated barley contains 4% moisture.

3 replicates/diet, and placed in electrically-heated batteries with raised wire floors. Continuous lighting was provided and feed and water supplied *ad lib*. A basal diet of following percentage composition was used: 63.5 barley, 25.5 dehulled soybean oil meal, 5 herring fish meal, 1.75 dicalcium phosphate, 1.15 limestone, .3 salt, .11 DL-methionine, .025 manganese sulfate, and a vitamin mix supplying 2650 I.U. Vit. A, 660 I.C.U. Vit. D, 11 I.U. Vit. E, 8.8 mg riboflavin, 10 mg pantothenic acid, 33.2 mg niacin, .66 mg folic acid, 884 mg choline chloride, and 22 mg procaine penicillin/kilo diet. Experimental data from Tables II and III were statistically analyzed, using Duncan's(7) Multiple F Test.

Results. Microbial counts markedly increased when barley was water-treated, sterilized (dried) and water-treated, or sterilized and supplemented (inoculated) with fungal amylase (Table I). Average weight of chicks fed diets containing these barley samples correlates positively with count data. The relatively large counts obtained would not be predicted since an oven temperature of 70°C was employed. Perhaps microbial growth occurred because evaporative loss of moisture from barley lowered the internal temperature of the wet grain to less than 70°C during the first few hours of drying. The sterilized barley was probably contaminated during transfer from autoclave to drying oven, since no precautions were taken to prevent contamination. The fungal amylase supplement added to sterilized barley was not a sterile product and greatly increased counts.

When an inoculum of peptone washed culture (100 ml — 2×10^8 organisms ml) containing either *B. subtilis* (ATCC 9943) or the unidentified organism was added to sterile barley and allowed to stand 4 hours prior to drying (Table II), chick growth and feed utilization were significantly ($P < .01$) improved. Improvement of barley by inoculation with either organism was comparable to that of water-treating barley or enzyme supplementation. Since inoculum was added to extremely hot barley, growth of microorganisms was possibly the result of germination of spores. Addition of the unknown organism to sterile (wet-autoclaved) barley which was subsequently dried at 95°C did not improve growth.

Possibly the organisms used contributed enzyme(s) to the barley which in some manner acted to improve its nutritional value. A proteolytic enzyme has been crystallized from cultures of *B. subtilis*(8) which is partially effective in improving growth of birds fed barley(9). This improvement may occur during water-treatment since the unknown organism was isolated from barley itself.

Addition of a small (100 ml—2×10^7 organisms/ml) culture of the unidentified organism directly to dry barley diet, improved growth and feed efficiency (Table III). A significant ($P < .05$) growth response, similar in magnitude to that obtained with dry fungal amylase, was obtained when a large (400 ml—2×10^7 organisms/ml) culture was added. When dry diets containing water-treated barley or fungal enzyme were inoculated with the unknown organism, no significant improvement in growth was obtained. Autoclaving previously water-treated and dried barley in the presence of 25 or 125% added moisture did not lower its nutritional value for chicks. The nutritional value was not reduced by autoclaving. Therefore, it appears

that barley was improved prior to incorporation in the diet and improvement did not depend on microbially produced enzymes acting in the digestive tract of the chick.

Only 100 ml of 20-hr peptone washed culture significantly ($P<.01$) improved the nutritional value of sterile barley in Exp. 2 (Table II). Thus, it is highly probable that microorganisms grew in wet barley, perhaps producing enzymes, which could have acted on the barley substrate during drying. Exp. 3 (Table III) showed that 400 ml of nutrient broth culture gave a significant ($P<.05$) growth response, presumably a result of enzymes produced during incubation of the stock cultures.

The data demonstrate that microbial fermentation is effective in improvement of barley by water-treatment. This, however, does not eliminate the possibility that enzymes endogenous to barley may also play a role in improving the nutritional value of this grain by water-treatment. Both phenomena could conceivably take place simultaneously.

Summary. Water-treated barley taken from drying oven contained many more bacteria than untreated barley, even though drying temperature was 70°C. The nutritional value of sterilized barley inoculated with Gram-positive rod isolated from dust, or with *B. subtilis* (ATCC 9943) and dried at 70°C was highly significantly improved. Large inoculations of the organism isolated and grown in nutrient broth elicited a significant growth response. This response was not as great as that obtained by water-treatment or addition of fungal enzyme to the feed. Autoclaving did not lower nutritional value of water-treated barley. This fact indicates that the change(s) responsible for improvement was effected prior to feeding and was not due to presence of an enzyme(s) produced during treatment process that later acts on barley in the digestive tract of the chick.

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Antibody Response to Influenza Vaccines Combined with Hexadecylamine. (25459)

ALLEN F. WOODHOUR, KEITH E. JENSEN AND JOEL WARREN
Biologics Research Dept., Chas. Pfizer & Co., Terre Haute, Ind.

Certain lipids, including cholesterol, palmitic acid, stearic acid, and longer aliphatic amines, such as hexadecylamine (HDA) have been shown by Youngner and Noll to possess an affinity for several myxoviruses(1,2,3). Such virus-lipid complexes increase antigenicity, in an as yet unknown manner, to a level greater than that of equal amount of virus alone. This reaction is also notable in that large amounts of viral antigen can be adsorbed to a given quantity of HDA(3). These observations have an immediate bearing on the problem of enhanced potency of influenza vaccines. In an earlier study we reported preliminary observations on cholesterol containing vaccines(2). The following report describes the responses of man and animals to formalin-inactivated influenza viruses mixed with hexadecylamine, since, in comparative adsorption experiments, this compound ex-

hibited high affinity for a wide range of influenza strains.

Materials and methods. Hexadecylamine, $(CH_2(CH_2)_{15}NH_2)$ technical grade, was obtained from Eastman Kodak Co., Rochester, N. Y., as a waxy, white solid, insoluble in aqueous solutions. After thorough grinding with mortar and pestle and subsequent sifting through triple layers of surgical gauze, slurries were prepared by slowly adding influenza vaccines to finely ground lipid and further grinding the combination until a fine suspension resulted. Since HDA separates from suspension, it was necessary to agitate the preparation just before use to ensure homogeneity. Polyvalent influenza vaccines employed were 4-strain "civilian" (Pfizer lot #80431) and 6-strain "military" (Pfizer lot #88305) formulae containing 500 and 1000 CCA (chick cell agglutinating units)/ml, respectively.

Results. HDA in concentrations greater than 5 mg/ml was irritating when injected subcutaneously into guinea pigs. Therefore, to determine minimal amount of lipid that would enhance vaccine potency, varying amounts of HDA from 5 mg/ml to .1 mg/ml were combined with constant amount of 4-strain influenza vaccine and guinea pigs were injected with 1 ml of the mixture by intramuscular route. Animals were bled at 2, 4, and 8 weeks thereafter and sera assayed for hemagglutination-inhibition (HI) antibody titers using periodate and trypsin to destroy non-specific inhibitors. Levels were determined for all 4 strains in the vaccine, but only those for B/GL strain are illustrated as typical in Fig. 1. Vaccine mixed with .5 mg/ml or more HDA elicited higher geometric mean titers than found with control vaccine.

Toxicity of HDA was studied in mice, guinea pigs, monkeys, and subsequently in man. Although injection of 1 mg/ml in guinea pigs resulted in focal deposit of HDA which persisted for several weeks, lesser amounts were tolerated with no gross reaction. Histological examination of site of subcutaneous injection in guinea pigs and monkeys at intervals to 4 weeks revealed a transient inflammatory reaction of only slightly greater intensity than resulted from injection of influenza vaccine alone. After 21 days, the site of injection was difficult to find and fibrosis or abscess formation has never been observed in several hundred guinea pigs.

Toxicity studies in man were begun by injecting HDA subcutaneously or intramuscularly at 48-hour intervals using following increasing dosages: .01, .02, .05, .10 and .50 mg. Thus, each of 20 volunteers received a total of .68 mg. The material was well tolerated with no local or systemic reaction.* In further human trials, none of 60 men injected with .5 mg/ml alone or mixed with influenza vaccine exhibited any local or generalized ill effects or manifested any increased sensitivity to egg protein when given a repeated dose of vaccine after a 90-day interval.

To determine adjuvant action with a wider variety of influenza strains, HDA was incorporated into a 6-strain vaccine in concentration of .5 mg/ml. Fig. 2 illustrates geometric mean titers in sera from groups of 20 guinea pigs vaccinated intramuscularly with this preparation and assayed for HI antibody at bi-weekly intervals to 8 weeks. With the PR 301 and PR8 antigens, peak titer was reached in 2 weeks contrasting with results obtained when measuring with the Asian and 2 B strains, where maximal titers were not obtained until after 4 weeks. The response to swine antigen was particularly disappointing, even though the vaccine contained 100 CCA units, a concentration shown to be satisfactory in eliciting antibody in the mouse potency assay for this biologic. Therefore, for reasons unknown at present, the swine component appeared to be a poor antigen in guinea pigs.

Influenza vaccine containing HDA not only elicited higher antibody titers than control vaccine but did so within a relatively short period of time. The elevated level did not persist, although titers in the test group were higher than controls for at least 8 weeks. Similar results have been obtained repeatedly in other experiments utilizing 4-strain vaccine. Typical data are summarized in Fig. 3 where guinea pigs were again the test species. Geometric mean titers found with 4 strains were cumulatively plotted so that a clearer com-

* We thank Dr. D. Iezzoni, Clinical Research Dept., Chas. Pfizer and Co., who conducted this study.

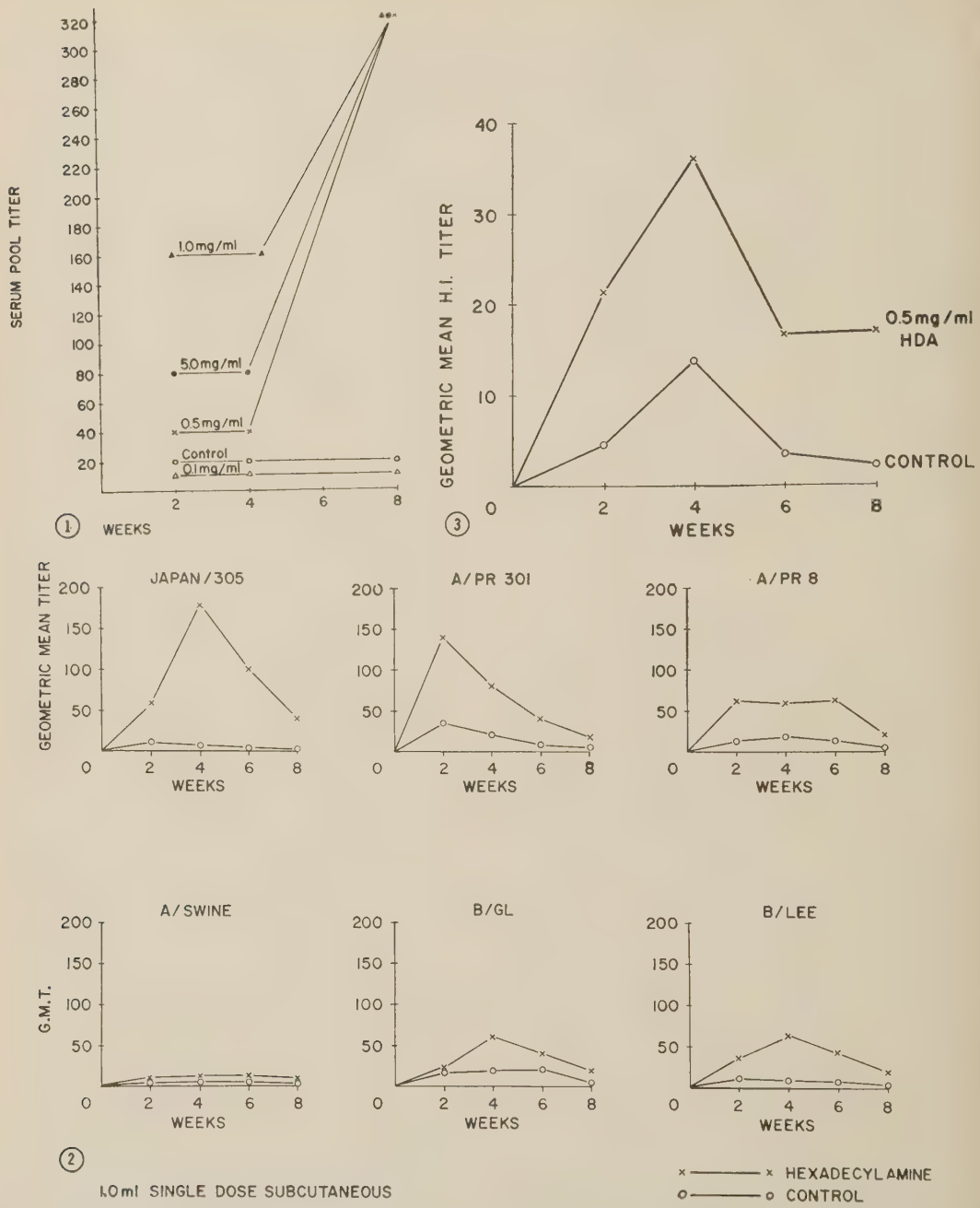


FIG. 1. Guinea pig responses to B/GL strain in combination with various amounts of hexadecylamine.

FIG. 2. Guinea pig antibody responses to 1000 CCA six strain influenza vaccine with or without hexadecylamine.

FIG. 3. Guinea pig combined antibody response to 500 CCA four strain influenza vaccines.

parison of the over-all response to a vaccine with and without added HDA could be obtained. The average titers of animals receiving HDA adjuvanted biologic remain 3- to 4-fold greater than control vaccine at each bleeding interval.

In experiments cited thus far, HDA was added directly to influenza vaccines, and the

TABLE I. Guinea Pig Antibody Titers Following Injection of Monovalent Influenza Vaccines Adsorbed to Hexadecylamine.

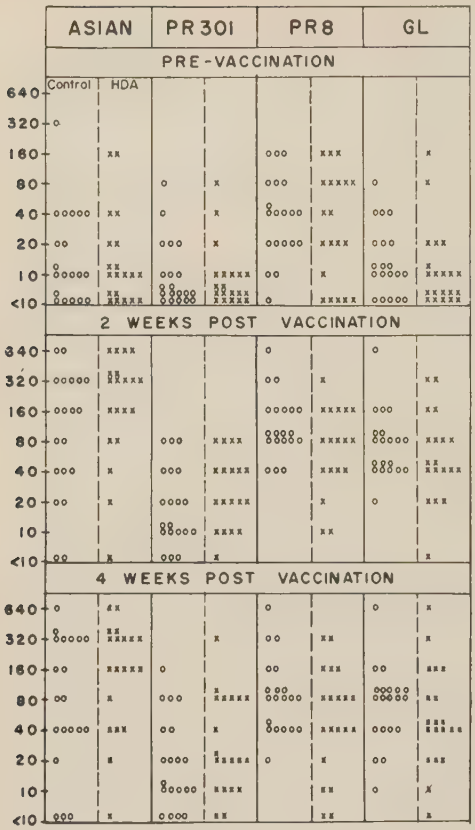
Type	HA, units/ml	Preparation	Conc. HDA/mg	Wk post-vaccination 2	4	7
Swine	639	HDA	.5	23*	93	59
	"	C†	.0	1	9	6
	148	HDA	.1	2	12	6
	"	C	.0	2	2	2
	30	HDA	.02	1	1	1
	"	C	.00	1	1	2
	6	HDA	.004	1	1	1
	"	C	.00	1	1	1
B/GL	494	HDA	.5	69	87	64
	"	C	.0	14	7	8
	99	HDA	.1	10	27	18
	"	C	.0	3	3	4
	20	HDA	.02	1	1	3
	"	C	.00	3	2	3
	4	HDA	.004	1	1	2
	"	C	.000	1	1	1
Asian	890	HDA	.5	24	160	50
	"	C	.0	16	29	21
	178	HDA	.1	5	95	11
	"	C	.0	2	5	2
	37	HDA	.02	1	6	2
	"	C	.00	3	8	9
	8	HDA	.004	1	4	1
	"	C	.000	1	7	2

* Reciprocal of geometric mean titer.
† C = Control.

actual amount of virus bound to the amine was not determined. To ascertain whether vaccine with only HDA-bound antigen might be superior, HDA was saturated with inactivated monovalent influenza concentrates and the hemagglutinating (HA) units/ml of adsorbed virus calculated. Vaccines were then diluted so that 1. ml contained .5, .1, .02, and .004 mg/ml HDA. Such dilution also reduced the number of hemagglutinating units by 5-fold increments. Comparable control vaccines without HDA were also prepared. Guinea pigs were injected intramuscularly with 1 ml of a given preparation, bled at intervals of 2, 4, and 7 weeks and sera assayed for HI antibody titer. Data for swine, B/GL, and Asian strains are presented in Table I. Addition of HDA to monovalent influenza vaccines in concentrations of .1 mg/ml or greater enhanced potency, while below that level adjuvant action was not obtained. Thus, it is clear that injection of 600 HA units firmly bound to HDA provided a pronounced adjuvant effect, even with the swine strain, a virus which had given poor results in previous experiments. It appears that when HDA-bound antigen is used as vaccine, a more striking enhancement occurs than with injection of mixtures of lipid-adsorbed and free virus antigens.

Results of studies in human volunteers with a single one ml subcutaneous injection of commercial 4-strain influenza vaccine to which .5 mg/ml HDA was added are presented in Fig. 4. The distribution of antibody titers against the vaccine strains is shown for groups of 20 adult males with serum specimens obtained pre-vaccination, and at intervals of 2 and 4 weeks post-vaccination. The dots indicate subjects who received control vaccine and crosses are for those who were given vaccine with HDA. At 2 weeks, a slight enhancement of titer was observed with the Asian component, less with the PR301, none with PR8 and B/GL strains. A similar relationship held at 4-week bleeding interval. In light of the results obtained in animal trials, this pattern of human response was totally unexpected. The same vaccine preparation was, therefore, retested in guinea pigs and sera assayed at similar bleeding intervals. As in previous experiments with this animal host, a pronounced adjuvant effect was again observed at 2 weeks after vaccination, and this enhancement was further evident in the 4-week sera (Fig. 5). This difference between animal and human response appears to be related to prior experience of human subjects with influenzal antigens since many of the men studied were previously sensitized to each of the 4 vaccine strains.

Discussion. Combination of HDA with influenza vaccines does increase antigenic potency; however, our results illustrate a factor of significance in comparative studies of adjuvants in animals and man. When the test subject has had previous sensitization to a given antigen, such as is often the case in adults, the response may be only slightly increased. In contrast, enhancement will be observed with HDA-virus mixtures in a host undergoing primary immunization. The adjuvant effect appears to be more pronounced



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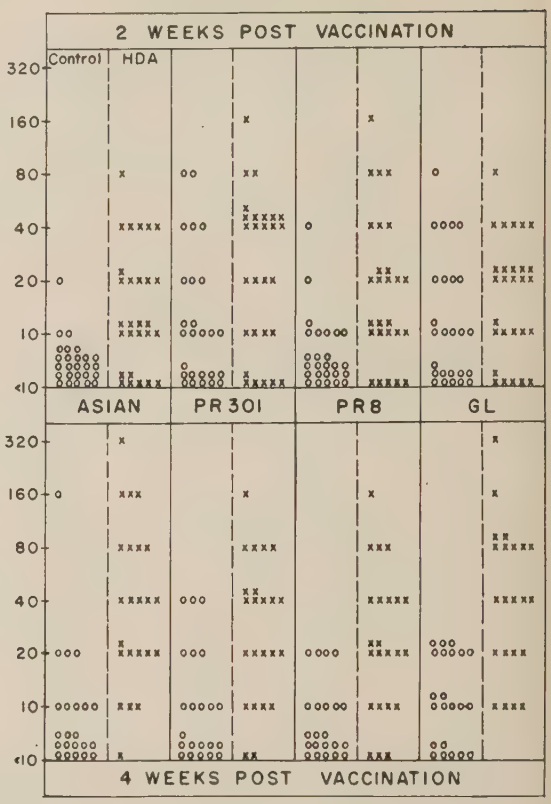

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FIG. 4. Antibody responses of adults to influenza vaccine with hexadecylamine.
FIG. 5. Antibody responses in guinea pigs to influenza vaccine with hexadecylamine.

when virus antigens are firmly bound to HDA. Studies are now in progress of responses in sensitized guinea pigs and men with virus-coated HDA vaccines.

Rapid antibody production can be an important factor in the control of influenza when new vaccines are being used to combat rapidly spreading new antigenic variants such as was the case in the pandemic of 1957. For this reason, the accelerated responses induced by myxovirus adsorbed to lipid is a valuable feature of the combination.

Summary. Hexadecylamine in levels up to .5 mg/ml is well tolerated by man and animals. A single injection into guinea pigs of influenza vaccines containing this material results in rapid production of antibody levels which exceed those elicited by control vaccines. Preliminary results in man suggest that adjuvants of this nature may be most efficacious in a population receiving primary influenza experience.

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Prevention of Experimental Allergic Encephalomyelitis with 6-Mercaptopurine.* (25460)

Leon W. Hoyer,† Richard M. Condie‡ and Robert A. Good
Pediatric Research Laboratories, Variety Club Heart Hospital, Univ. of Minnesota

Despite extensive investigation, the mechanism whereby administration of nervous tissue in adjuvants results in production of experimental allergic encephalomyelitis (EAE) remains to be conclusively demonstrated(1). Extensive indirect evidence suggests that EAE has, in a poorly understood manner, an immunologic basis(1,2). Although development of EAE has been prevented by a number of agents which interfere with the immune response, repeated failures to achieve passive transfer of the disease with serum or cells from involved animals to normal animals raises a question concerning other evidence indicating that EAE is an immunological disease. Studies by Sterzl(3) on use of 6-mercaptopurine to influence the immune response, and the more recent demonstration by Schwartz(4) that 6-MP will prevent production of circulating antibody in response to first parenteral injection of simple protein antigens, would seem to provide another approach to elucidation of the pathogenic mechanisms in development of EAE. This paper reports results in which treatment with 6-MP prevented development of EAE.

Material and methods. Rabbits employed were albino hybrids of both sexes, weighing 2-3 kg, obtained from a single breeder and kept in the laboratory for at least 2 weeks prior to experimentation to insure a disease-free stock acclimated to laboratory conditions. 6-MP§ was dissolved in 1N NaOH (0.15 g/ml) and the solution kept under refrigeration. Fresh solution was made up every other day. All injections of 6-MP were made into marginal ear vein. The encephalitogenic agent was whole rabbit spinal cord, freed of meninges and large blood vessels by being forced through stainless steel tissue press containing #80 mesh copper wire screen, and emulsified in a modified Freund's adjuvant: 20 g wet weight spinal cord; 10 ml complete Freund's adjuvant (Difco); 500 mg ground, killed, dried Mycobacterium butyricum; 10 ml Bayol F; and 2 ml Arlacel A. Rabbits received 0.1 ml of this emulsion intradermally in each hind footpad. Five groups of animals were used. Group 1a received 12 mg/kg/day, starting 2 days prior to injection of spinal cord-adjuvant emulsion and continuing through 18th day after injection. Group 1b (12 mg/kg/day), Group 2 (9 mg/kg/day), and Group 3 (6 mg/kg/day) received 6-MP daily, starting day of injection of spinal cord-adjuvant emulsion and continuing through 18th day after injection. Each experimental group contained 10 animals. A group of 20 animals served as controls and received no injections with 6-MP.

Results. Although EAE may be manifested by paralysis, tremors, convulsions, coma, and death, the most consistent clinical manifestation of this disease in rabbits is paralysis. Indeed, in our experience paralysis is a most satisfactory index for occurrence of EAE in this species. In observations of EAE in more than 500 rabbits, paralysis has never been present without confirmatory histologic lesions, including perivascular cuffing and evidence of demyelination or necrosis within the central nervous tissue. On the other hand, in almost all animals with demonstrable histologic lesions, paralysis of some degree has been observed. Alvord, Magee, Kies, and Goldstein(5) report that in the guinea pig, as well, paralysis is almost always associated with microscopic lesions of the central nervous system. Consequently, in this study, paralysis was used as primary index of presence or absence of disease. Animals were observed daily

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† National Science Foundation Medical Student Summer Research Fellow.

‡ Research Fellow of Child Development Study Group, Univ. of Minnesota.

§ "Purinethol" 6-Mercaptopurine was generously provided by Burroughs Wellcome & Co.

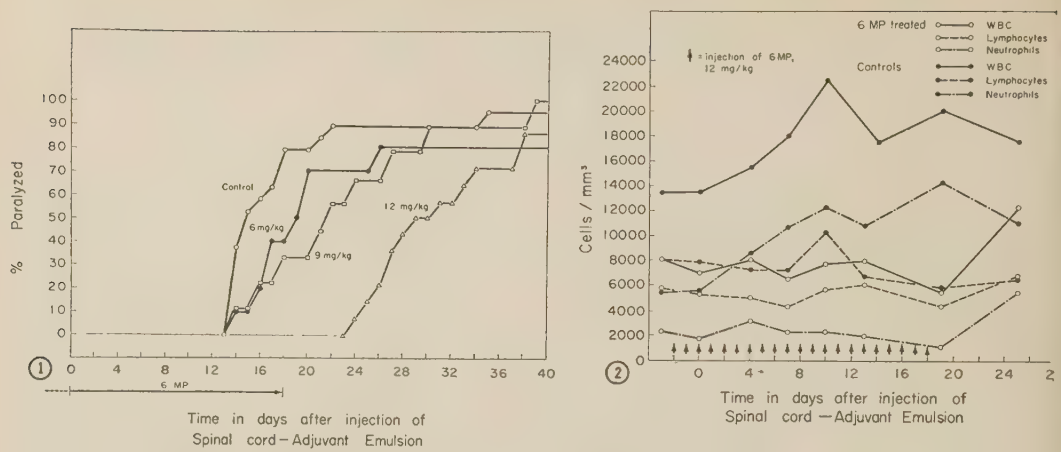

FIG. 1. Effect of 6-MP on experimental allergic encephalomyelitis.
FIG. 2. Effect of 6-MP on leukocytes in rabbits stimulated with a spinal cord-adjuvant emulsion.

and were considered to have the disease only if definite signs of paralysis were observed in either the front or hind quarters. In a number of animals dying of EAE, histological examination revealed the characteristic lesions (1).

Fig. 1 shows the time course of development of paralysis, Groups 1a and 1b being plotted together. Eight of the 60 animals in Groups 1-3 were not used to determine percentages of paralyzed animals given in Fig. 1. These animals include: one of the control group which died on fourth day after injection of spinal cord-adjuvant emulsion; one animal of Group 3 and 3 animals of Group 1 which died immediately following heart puncture; and 3 animals of Group 1 which died of apparent toxicity due to 6-MP. (All died on nineteenth day after injection of spinal cord-adjuvant emulsion.) These animals showed no evidence of paralysis prior to death. Although lower dosages of 6-MP (6 and 9 mg/kg/day) delayed appearance of the disease, they did not prevent appearance of paralysis even while they were being given. However, when 6-MP was given in a dosage of 12 mg/kg/day, it completely prevented development of the disease during drug administration.

Because previous studies had indicated that animals given 12 mg/kg/day of 6-MP remained in apparent good health for 18-20 days, and then began to sicken and die if injections of 6-MP were continued, administration of the drug was discontinued on eighteenth day in this experiment. After discontinuation of the drug, and following a latent period of 7-18 days, the rabbits developed paralytic encephalomyelitis which differed in no way from the disease observed in controls. It is notable, however, that the interval between discontinuation of 6-MP and appearance of paralysis was somewhat shorter than the interval between injection of spinal cord-adjuvant emulsion and appearance of paralysis in control animals. It is unlikely that 6-MP merely masked clinical manifestations of EAE in this group, for after drug treatment was discontinued at least 7 days elapsed before paralysis due to EAE was observed in these animals.

Effect of 6-MP on circulating leukocytes. Administration of 6-MP to animals in Group 1a produced slight depression in total number of circulating leukocytes (Fig. 2). Continued administration of 6-MP to animals not stimulated with an antigen, results in a severe leukopenia(6), but this was prevented in our experiments by the stimulation provided by injection of the spinal cord-adjuvant emulsion. The hematologic data for a group of control rabbits are also given in Fig. 2. It is apparent that the increase in total number of leukocytes associated with EAE is due to increase in numbers of neutrophils. In these control animals, as well as in 6-MP-treated animals, the number of lymphocytes in peri-

pheral blood remained nearly constant. 6-MP in these doses prevents the increase in circulating neutrophils and produces a mild neutropenia.

Discussion. A number of studies have shown that it is possible to inhibit or diminish the severity of EAE. These have included excision of lymph nodes draining the injection site, irradiation of these nodes prior to injection of the spinal cord-adjuvant emulsion, prior injections of aqueous suspensions of nervous tissue in newborns(7) and adult animals(8) and treatment with salicylates(9), ACTH and cortisone(10,11,12).

Our experiment indicates that a third type of chemical, a nucleic acid antimetabolite, will also prevent occurrence of EAE while it is being given. The effect of 6-MP on immune response is probably a result of its antimetabolic properties. Schwartz *et al.*(4,13) found that antibody production is inversely related to dose of 6-MP administered, and interpreted this to mean that the drug specifically interferes with a first-order chemical reaction rather than having a general cytotoxic effect similar to that of nitrogen mustard and X-irradiation. Whether DNA metabolism or RNA metabolism is affected is not clear at present. Interference with either induced proliferation or differentiation of cells may be the basis of this effect.

Only very large doses of 6-MP prevented occurrence of encephalomyelitis. However, the majority of rabbits so treated tolerated the drug quite well and, following its discontinuation, were healthy enough to express the disease in its characteristic form. Certainly the rabbits were not debilitated during treatment with 6-MP and the effects observed cannot be attributed to drug toxicity *per se*. It is of interest that, even with the intensive stimulus represented by injection of spinal cord-adjuvant emulsion administered, some delay in development of EAE was observed with non-toxic doses of 6-MP (6 mg/kg and 9 mg/kg).

While the immunological nature of EAE seems well established, the nature of the hypersensitivity involved is not yet clear. The effect of 6-MP on delayed hypersensitivity has not been reported, but Schwartz *et al.*(13) were able completely to suppress formation of circulating antibody by continued injections of 6-MP. Whether or not 6-MP will aid in identifying the nature of the hypersensitivity involved in pathogenesis of EAE, remains a question to be answered by further experimentation. It is clear, however, that EAE can be suppressed by 6-MP without a reduction in circulating level of lymphocytes, the cell type most usually implicated in the mechanism of delayed hypersensitivity.

Summary. Experimental allergic encephalomyelitis is prevented during administration of 12 mg/kg/day of 6-Mercaptopurine. The number of lymphocytes in peripheral blood is not reduced during this period in which 6-MP prevents EAE, although the number of neutrophils is reduced by half.

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Effects of Posterior Hypothalamic Lesions on Sexual Maturation of Immature Female Albino Rats. (25461)

Alan Corbin* and B. A. Schottelius
Dept. of Physiology, College of Medicine, State University of Iowa, Iowa City

Neuroendocrine investigations demonstrated that hypothalamic lesions will impair mechanisms for normal maturation of the prepuberal rat. Bogdanove(1) and Donovan (2) have shown that precocious sexual development can occur in immature female rats with anterior hypothalamic lesions. Such effects may be due to either release from a gonadotropic inhibiting system, or activation of a gonadotropic stimulatory mechanism. Although the underlying processes involved in initiation of puberty are relatively obscure, the present belief is that lack of hypothalamic stimulation delays puberal onset, since, in the sexually infantile rat, the pituitary is not too immature for, nor its target organs refractory to, gonadotropic hormone secretion. Transplantation of immature rat pituitaries to base of brain of their hypophysectomized mother restored cyclic estrus function before puberty in the donors would have been effected(3), indicating that prepuberal hypophysis awaits hypothalamic stimulation. Investigation of the possibility that posterior hypothalamic damage may cause a delay in onset of puberty is the object of this study.

Methods and materials. Forty-five, 21-day old albino female rats (Holtzman) were divided into 4 groups. Group A (12 animals) was a normal control group, Group B (12 animals) was the sham operated control group, Group C (11 animals) consisted of lesioned subjects, and Group D (10 animals) was a normal control group sacrificed at 21 days of age. Animals were allowed tap water and Purina laboratory chow. *ad lib.* Body weights were recorded every 5 days. All operations were performed between days 21 and 25. Under Nembutal anaesthesia (2 mg/50 g rat, I.P.), a 23 ga wire electrode was inserted, according to Krieg(4) coordinates 53-56, 83-84, to a depth of 0.5-1 mm from base of skull. Direct current of 5 ma for 15 sec was employed to bilaterally destroy the area from the mammillary body to the ventromedial nucleus. As soon as vaginae opened, smears were taken for 3 to 4 weeks to identify cycling activity. At the first such smear, one rat from each of Groups A and B, and 3 from Group C was placed in a cage with a virile, 100 day old male, for mating. All rats were observed for behavioral changes. Groups A, B, and C were sacrificed at 65 days of age. Fresh weights of pituitaries and reproductive organs (total weight of ovaries + oviducts + uteri + vagina) were recorded. Statistical analysis of data was by Student's "t" test. A probability of ≤ 0.05 was accepted as statistically significant.

Results. Histological examination of lesioned brains revealed extensive destruction of the area from mammillary body to VMN. Results are summarized in Tables I and II. Animals with hypothalamic lesions showed a definite inhibition of reproductive organ growth. Average reproductive organ weight of 5 lesioned animals whose vaginae never opened was 93.9 mg, less than $\frac{1}{5}$ the average recorded for normal controls. Although pituitaries of lesioned animals were considerably smaller than those of normal controls, they were comparable in weight to those of the sham operated group, making it difficult to interpret reproductive organ size in terms of pituitary weight alone.

Vaginae of Groups A and B opened between days 34 and 35, with a cycling pattern established, on the average, 6 days after opening. In contrast, vaginal opening of Group C was delayed until the 52nd day. This delay is especially significant since the group includes 5 animals (Group C_2) whose vaginae were still closed at autopsy. These animals had the smallest reproductive organ and pituitary weights of the entire lesioned group. All Group C animals exhibited abnormal deposits

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TABLE I. Means and Standard Errors of Body Weights, Reproductive Organs, Hypophysis, Vaginal Opening, and Cycling in Normal, Sham Operated, and Lesioned Female Albino Rats.

	No. of rats	Body wt (g)		Reproductive organ wt (mg)		Hypophysis wt (mg)		Day vagina opened	Cycling began, days post vaginal opening
		21 days	65 days	21 days	65 days	21 days	65 days		
Group A, normal control	12	48.0 ± .8	191.5 ± 3.2		552.5 ±65.1		8.12 ±1.14	35.0 ± .6	6.0 ±.4
Group B, sham control	12	42.0 ±1.2	188.9 ±10.4		604.6 ±75.8		3.70 ±1.00	34.0 ±1.4	6.0 ±.4
Group C, lesioned	11	42.0 ±1.7	180.9 ± 7.4		213.4 ±60.9		2.01 ± .33	52.0 ±3.5	Anestrous
Group C_1, lesioned vaginae opened	6	42.0 ±3.0	183.5 ± 4.0		332.0 ±29.0		2.28 ± .77	40.0 ±2.1	"
Group C_2, lesioned vaginae never opened	5	42.0 ±4.2	178.8 ±23.6		93.9 ±13.8		1.75 ± .50	Unopened (at 65 days)	
Group D, normal controls 21 days old	10	42.0 ±1.4		66.64 ±1.35		.823 ±.177			

of adipose tissue in which kidneys, adrenals and unvascularized reproductive organs were deeply embedded. That such accumulation of fat exceeded that of the 2 control groups, and that large lower abdominal bulges were evident, suggested imminent obesity. One lesioned animal exhibited definite obesity, weighing 231 g. Females from Groups A and B became pregnant, bearing an average of 12 fetuses. The 3 animals from Group C when tested never became pregnant.

Of 11 animals in Group C, 5 had vaginae that never opened, 5 were diestrus and gave continuous leucocyte smears, 1 was in constant estrus, and gave continuous cornified smears (this animal failed to mate).

Marked behavioral alterations were observed in the lesioned animals. Post-operatively, the rats became extremely vicious and

TABLE II. Table of "t" Values for Normal, Sham Operated and Lesioned Female Albino Rats.

Groups compared	Reproductive organ wt	Body wt (65 days)	Hypophysis wt	Day of vaginal opening
A & B	.52†	.024†	2.9 *	.6 †
A & C	3.77*	.16 †	5.00*	4.5 *
B & C	4.00*	.05 †	1.50†	4.5 *
A & C_1	3.10*	1.57 †	4.30*	2.62‡
A & C_2	6.60*	.022†	4.16*	
B & C_2			1.71†	

* $P \leq .01$ † $P \geq .05$ ‡ $P \leq .02$

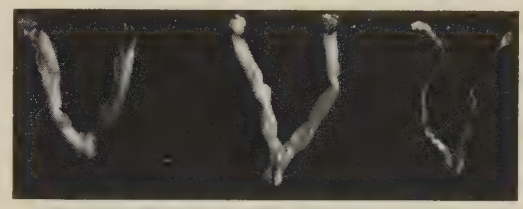

FIG. 1. Comparison of reproductive organs of normal control (left), sham-operated (center) and lesioned rat (right) at 65 days.

belligerent. Two of the weaker surviving animals were eaten alive. Continuous hostility was exhibited up to time of sacrifice. Pain and post-operative discomfort were ruled out as possible stimuli since the sham operated group displayed no such behavior, and normal rats of such a young age are quite docile and inquisitive. Food intake of lesioned animals was reduced during the first 2 post-operative weeks. Definite hyperphagic behavior was indicated by day 50, at which time the bulging, pear shaped lower abdomen became evident.

Discussion. Fig. 1 shows the striking difference in reproductive organs of a normal control, sham operated and effectively lesioned animal. The reproductive organ immaturity and prolonged vaginal closure infers that the initiating mechanism for estrogen secretion was absent. Reproductive organ growth and maintenance of functional estrous cycling, as well as vaginal opening "on time," are depen-

dent upon this particular ovarian hormone. Furthermore, the fact that lesioned animals whose vaginae opened after considerable delay exhibited a constant anestrous state, suggests that the hypophysis was incapable of elaborating or secreting those hormones responsible for initiation and maintenance of sexual cycling.

On the other hand, rats with anterior hypothalamic lesions have exhibited precocious puberty(1,2) and persistent estrus(5,6,7). Correlation of all observations suggests the possible existence of a dual "gonado-hypophytropin" controlling system: 1) an inhibitory mechanism located in the anterior hypothalamus, and 2) a stimulatory one located in the posterior hypothalamus. Activity of these 2 hypothetical "sex centers" should determine net gonadotropic activity of the hypophysis, and influence time of puberal initiation. The effects of circulating gonadotropic and ovarian hormones upon hypothalamic function must also be considered, although further discussion of such feedback mechanisms is beyond the scope of this study. In view of previous evidence, the hypophysis is, theoretically prepared to produce its gonadotropins at a prepuberal age. The reason for puberty not normally occurring earlier may be due to a differential immaturity of the hypothalamus itself.

Physiological evidence for ventromedial nuclei destruction was indicated by the obese patterns in these animals, confirming reports of Brobeck(8). It is interesting to note the similarity between delayed puberty, sexual infantilism and obesity resulting from posterior hypothalamic destruction and the characteristic signs of Frohlich's syndrome. In effect, Frohlich's syndrome has been experimentally produced in these animals.

Summary. Eleven 21-day old female albino rats (Holtzman) were subjected to bilateral lesions of the area from the mammillary body to the ventromedial nucleus. These animals showed definite signs of delayed puberty, reproductive immaturity, viciousness and imminent obesity. Five had vaginae that never opened. It appears evident that in the prepuberal rat, factors (neurosecretory substances), which prod the pituitary into a functional secretory state, and which permit the mammal to attain reproducive maturity, are not present, or, are in a state of preparation. The results indicate the importance of hypothalamic function to puberal onset, and to the structural and physiological success of the reproductive-sex complex.

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Effect of Methionine upon Ethionine Intoxication of the Rat.* (25462)

ROBERT J. STEIN, GEOFFREY KENT, TIBOR BARKA AND HANS POPPER
Division of Experimental Therapy, Abbott Labs., North Chicago, Ill., and Dept. of Pathology, Mount Sinai Hospital, N. Y. City

Ethionine is an analogue and metabolic antagonist to methionine(1,2,3), and ample evidence has been presented that the features of ethionine intoxication including cancer are prevented by administration of methionine(4). Nevertheless, the possibility of a toxic effect

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of ethionine is not excluded. To supplement available information, the effects of various ratios of ethionine and methionine in the diet upon the subacute hepatic changes in the rat liver were studied. Particularly the effects of such ratios upon liver cell injury and ductular cell reaction were investigated, as they are the 2 most distinctive features of sub-acute ethionine intoxication(5).

Methods. Female Sprague-Dawley rats, weighing approximately 175 g, were placed in individual cages and kept on synthetic diet containing vitamin free casein, 160 g; corn oil, 50 g; sucrose, 750 g; salt mixture U.S.P., for depletion diet, 40 g/kg; and adequate vitamin supplements, except for riboflavin, 4 mg; and choline hydrochloride, 30 mg. The rats were divided into 16 groups each containing 10 animals. Five of these groups received each supplements of 0.5%, 0.75%, and 1% ethionine, respectively, whereas the last group served as overall control. In each of the 3 experimental groups, one subgroup received no supplement of methionine, whereas the other 4 in each group received supplements varying from 0.4 to 1.4% methionine (Table I). After 35 days all rats were sacrificed, and liver tissue was fixed in buffered 10% formalin and some in Carnoy's fixative. Paraffin sections were stained with hematoxylin-eosin, Gomori's silver impregnation for reticulum and subjected to Schiff's periodic acid reaction after removal of glycogen by diastase. Degrees of hepatic cell degeneration, of ductular cell proliferation, and reticulum increase were arbitrarily graded by 2 independent observers from 0 to 4 plus, without knowledge of the experimental history according to criteria previously used(6).

Results. Rats on ethionine alone exhibited severe damage to parenchymal cells of the liver associated with necrosis and regeneration of single cells (Fig. 1). Liver cells were often arranged in small groups as a result of formation of several cells thick plates. Between liver cell plates ductular cells accumulated frequenty without apparent arrangement into tubules. They were surrounded by mononuclear cells and segmented leucocytes, the combined lesion of ductular cells and inflammatory cells being designated interstitial cell reaction. The argentophil reticulum fibers were conspicuously increased, many of them arranged around ductules (Fig. 1). The described changes were increasingly severe as concentration of ethionine in the diet rose. In rats receiving methionine supplements slightly below ethionine levels, the hepatocellular changes were decreased in intensity. This became more pronounced as methionine supplements were raised to one-and-a-half or twice those of ethionine without, however, being completely normal. In contrast, ductular cell proliferation and the less regular accumulations of inflammatory cells were abolished by the smallest amount of methionine used. This also held true for the increase in reticulum fibers.

TABLE I. Arbitrary Grades (Average of 10 Rats per Group) of Histologic Features in Liver of Rats on Synthetic Diet for 35 Days with Varying Supplements of Ethionine and Methionine.

g/100 g of synthetic diet				
Ethionine	Methionine	Parenchymal degeneration	Interstitial cell reaction	Reticulum
0	0	±	0	0
.5	0	3-4+	2+	2+
"	.4	3+	0	0
"	.6	2+	0	0
"	.8	1+	0	0
"	1.0	"	0	0
.75	0	4+	3+	3+
"	.6	2+	0	0
"	.8	1-2+	0	0
"	1.0	"	0	0
"	1.2	±	0	0
1.0	0	4+	4+	4+
"	.8	1-2+	0	0
"	1.0	"	0	0
"	1.2	1+	0	0
"	1.4	±	0	0

Discussion. Intensity of histologic features of the sub-acute ethionine intoxication depends upon amount given and similarly, in confirmation of multiple observations, methionine suppresses the effects of intoxication almost completely. Thus, even high levels of ethionine failed to produce conspicuous changes if covered by increasing amount of methionine. The basal synthetic diet contains approximately 0.4 g methionine/100 g which has to be taken into consideration in evalu-

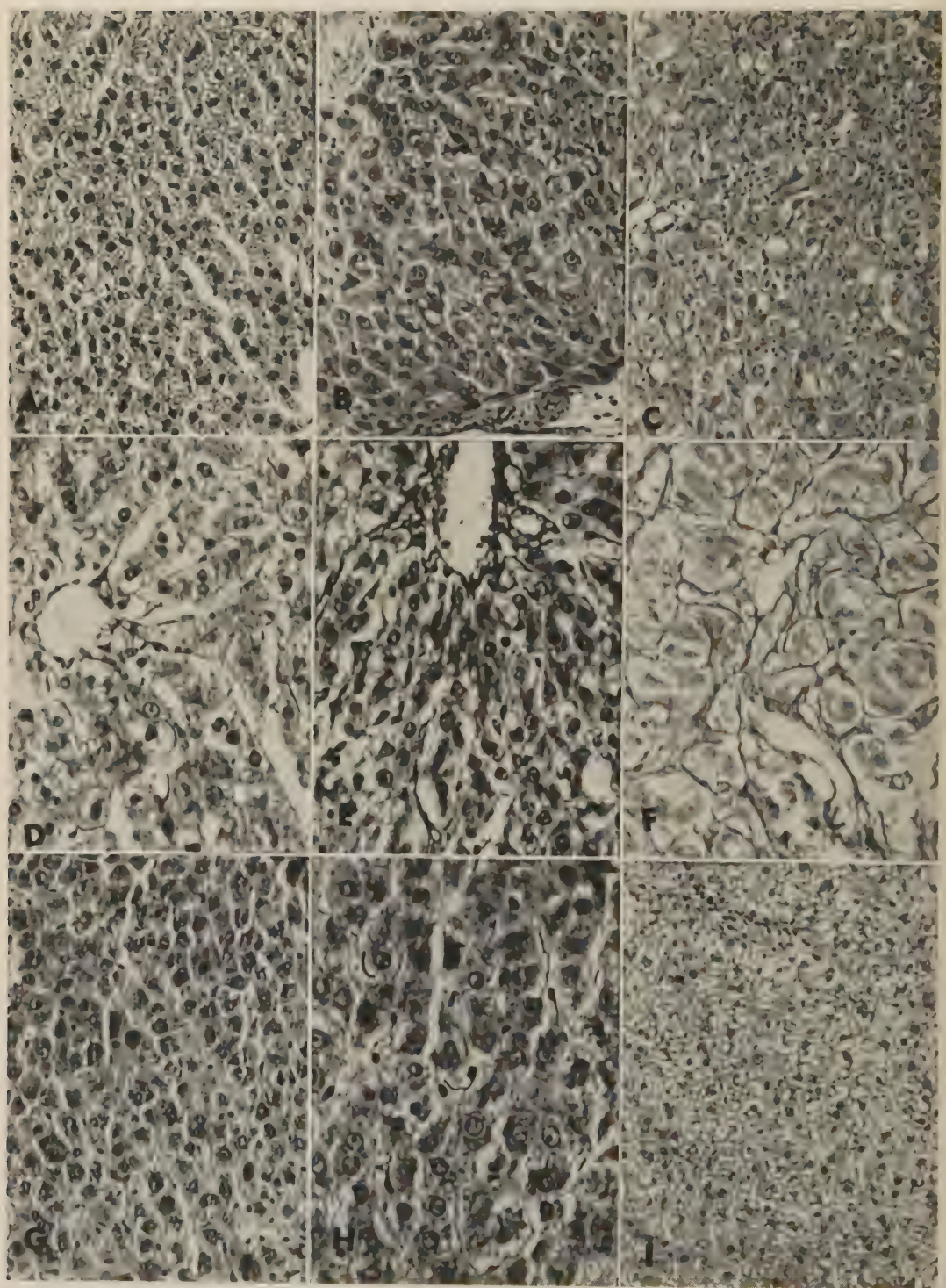

FIG. 1. Photomicrographs of livers of rats 35 days on various synthetic diets. A. Basal diet. Almost normal appearance of liver cells. HE 120×. B. 0.5% ethionine. Damage of liver cells and moderate interstitial reaction. HE 120×. C. 1% ethionine. Severe damage of liver cells and extensive interstitial reaction, composed of ductular and mesenchymal cells. HE 120×.

D. Basal diet. Small amount of reticulum. Gomori's silver impregnation 240×. E. 0.5% ethionine. Moderate increase of reticulum. Gomori's silver impregnation 240×. F. 1% ethionine. Conspicuous increase of reticulum, mainly arranged around ductules. Gomori's silver impregnation 240×. G. 0.5% ethionine and 0.4% methionine. Damage of liver cells without interstitial reaction. Compare with B. HE 120×. H. 0.5% ethionine and 0.4% methionine. Almost normal amount of reticulum. Compare with E. Gomori's silver impregnation 240×. I. 1% ethionine and 1.4% methionine. Slight irregularity of cytoplasmic staining of liver cells. No interstitial cell reaction. HE 120×.

ating methionine supplements. However, in view of the established differences in metabolic utilization between ethionine and methionine, it is difficult to calculate the effective ratio in the body. With this reservation it appears that higher absolute values of methionine are more effective in protection, in spite of the presence of larger quantities of ethionine. The results support the assumption of metabolic competition rather than toxicity for the ethionine effect.

Whereas on the same level of ethionine, the degree of liver cell damage reflects the amount of methionine supplemented, the interstitial cell reaction is prevented by all levels of methionine administered. Thus, in subacute ethionine intoxication the ductular cell proliferation and liver cell damage are dissociated in animals receiving methionine, similar to what may be observed when cortisone is administered to ethionine intoxicated rats(7). The discrepancy is particularly apparent in animals receiving relatively small methionine supplements. In animals receiving large supplements, routine histologic examination fails to show significant differences from control animals.

The parallelism in degree of interstitial cell reaction or ductular cell proliferation and increase of reticulum fibers is a further strong support for the concept that ductular cell proliferation is the most important stimulus for formation of fibers in ethionine intoxication in the rat(7,8).

Summary. Liver cell injury of subacute ethionine intoxication of the rat is accentuated by rising ethionine levels in the diet. Methionine supplements have an increasingly inhibitory effect depending on concentration. That effects of even high levels of ethionine may be almost completely eliminated by high methionine levels speaks for the role of ethionine as a metabolic competitor of methionine rather than as a primary toxic agent. Methionine inhibits ductular cell proliferation much more effectively than the injury to hepatic cells, thus dissociating both lesions. Ductular cell proliferation and fiber formation run parallel.

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Non-Specific Effect of Bromide on Conditioned Avoidance Behavior in Rats.* (25463)

Ira Rosenblum (Introduced by F. C. Ferguson, Jr.)
Dept. of Pharmacology, Albany Medical College, Albany, N. Y.

Bromide ion has been traditionally classified as a general central nervous system depressant and been used as a sedative and anti-epileptic drug. It has also been reported that bromide possesses ataractic or tranquilizing properties(1), suggesting another therapeutic use for it. Since there is little experimental information about the tranquilizing properties of bromide(2), this experiment examined the effect of bromide on conditioned avoidance response, using it as one criterion of tranquilizing activity(3).

Methods. The avoidance conditioning method described by Cook and Weidley(4) was used with modification. The test animal climbs a vertical pole to avoid unconditioned stimulus (US) (electric shock) delivered through a grid in floor of a box. The conditioning stimulus (CS) is a buzzer introduced for 5 seconds before shock. If animal climbs the pole during this period, no shock is delivered and this becomes the conditioned response (CR). Failure to mount the pole results in shock (3/second), for no more than 30 seconds. If animal climbs the pole during this period, then this becomes the unconditioned response (UR). A specific blockade of CR is indicated by failure to respond to CS though responding to the US. Non-specific blockade results from failure to respond to both CS and US. Male rats of Sprague-Dawley descent, weighing approximately 100 g at start of experiment, were used in groups of 10 for training. Twenty-five rats trained to the CR response were divided randomly into 5 groups. In each group, 1 animal served as control, and the other 4 received 100, 200, 300, 400 or 500 mg/kg respectively of Br^- administered as NaBr. Injections were made intraperitoneally, once daily, 5 days a week for 2 weeks to achieve a relatively stable blood Br^- level or until blockade of CR was noted.

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TABLE I. Effect of Bromide on Conditioned Avoidance Response.

Dose Br^- (mg/kg)	Blood Br^- (meq/l)*	No. rats tested	No. rats failing to respond CR	UR
Control		5	0	0
100	12.0 ± 2.3	4	0	0
200	18.2 ± 2.8	"	0	0
300	27.7 ± 5.3	"	0	0
400	35.0 ± 1.9	"	0	0
500	48.7 ± 1.7	"	4	4

* Mean $\pm$ stand. dev.

Testing was carried out each day approximately 4 hours after injection of NaBr. After 2 weeks of testing, or when blockade of CR was noted, a blood sample was taken by cardiac puncture under light ether anesthesia. The sample was analyzed for whole blood Br^- by the method of Brodie and Friedman(5).

Results. A wide range of blood Br^- levels were obtained (Table I) with group mean values ranging from 12 to 48.7 meq/l. At highest level of blood Br^- the CR was blocked. However this blockade was non-specific since the UR was also blocked. At this blood Br^- level all rats showed evidence of ataxia and loss of righting reflex, accounting for inability to respond to the US by climbing the pole. This type of blockade of both CR and UR occurs with administration of such general central nervous depressants as barbital and methylparafynol(4). Therefore, it seems that the effect of Br^- on the CR is similar to that of a general central nervous depressant rather than to that of a drug possessing specific tranquilizing activity.

Although only one method for assessing tranquilizing activity of drugs was employed, the same relationship between toxic and effective dose also occurs when Br^- is tested for ability to block audiogenic seizures in rats(6). In these tests a dose of 750 mg/kg of NaBr given intraperitoneally to rats was 100% successful in preventing seizures but caused incoordination in the animals. Since ability to

block audiogenic seizures has also been used to assess tranquilizing activity of drugs(3), it lends further support to the assumption that Br^- does not possess tranquilizing properties in non-toxic doses.

Summary. The effect of 5 doses of bromide was tested for effect on conditioned avoidance response in rats. Blockade of response occurred at dose of 500 mg/kg of Br^-, but this effect was non-specific, since it was accompanied by ataxia and loss of righting reflex.

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Inheritance of Capability of Human Esophageal Epithelial Cells to Grow with Diverse Carbohydrates.* (25464)

S. GAYLEN BRADLEY AND JEROME T. SYVERTON†

Dept. of Bacteriology and Immunology, University of Minnesota, Minneapolis

Several carbohydrates have been reported sufficient for growth of human cells in continuous culture in a medium composed of inorganic salts and dialyzed serum(1,2). Additionally, Chang(3) derived variants of HeLa and conjunctival cells able to grow in medium containing d+xylose, a usually insufficient sugar, as sole carbon-energy source. Previous work has not determined whether such capacity of human cells to grow with particular carbohydrates is constitutive or adaptive, or how such capacity is inherited. This paper is concerned with capacity of cells in culture to initiate and continue multiplication, given particular carbohydrates. Capacity of cultures to grow in particular carbohydrate media was employed as a measure of cell capacity. This measure was influenced by the proportion of capable or potentially capable cells in culture inocula, degree of capability of such cells, and ability of these cells to become established in surface culture. When capability to grow in a medium containing a specific carbohydrate was independent of continued addition of that carbohydrate during subculture, the capability was considered hereditary. If capability to grow in a particular medium was attenuated reversibly by substitution of another carbohydrate during subculture, the capability was considered adaptive, as opposed to constitutive. By definition, the presence of a constitutive enzyme and the power to make an adaptive enzyme are hereditary. The expression of an adaptive enzyme is dependent upon the substrate and therefore nonhereditary. Irreversible alteration of culture capability by presentation or withdrawal of the carbohydrate was viewed as evidence of selective enrichment of a culture with capable or incapable cells. This report is concerned with propagation of human esophageal epithelial cells in medium with various carbohydrates, derivation of substrains by serial culture in media containing fructose, d+xylose or d+galactose, and determination of heritability of capacity to grow in these carbohydrate media.

Materials and methods. Cells of human esophageal epithelium (Minn. EE 55-12-1) have been carried as a stable line for 4 years after establishment from tissue of a male infant with tracheo-esophageal fistula(4). *Medium* for growth of the parental (uncloned) EE strain contained 105 mM sodium chloride, 5 mM potassium chloride, 0.8 mM disodium

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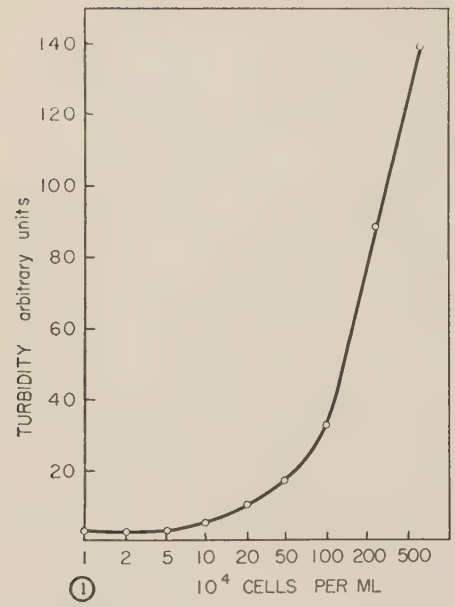
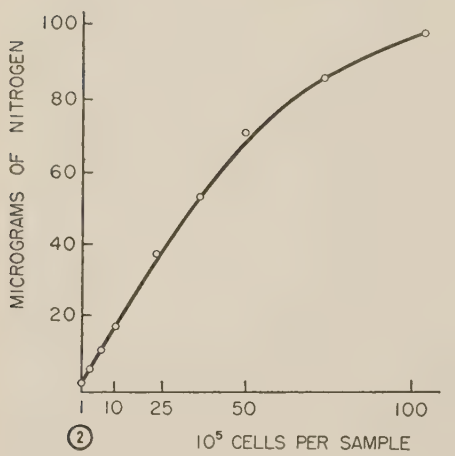
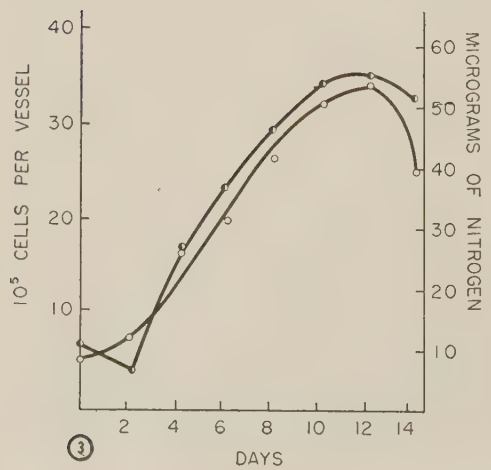






hydrogen phosphate, 0.3 mM sodium dihydrogen phosphate, 1 mM magnesium sulfate, 0.6 mM calcium chloride, 12 mM glucose, 0.002% (w/v) phenol red, 0.1% (w/v) Difco yeast extract, 10% (v/v) human serum, and sufficient sodium bicarbonate to adjust the pH to 7.6. Ten-times concentrated stock solutions of a) magnesium sulfate, calcium chloride and yeast extract, and b) remaining salts and phenol red, were sterilized by autoclaving; stock carbohydrate solutions were sterilized by Selas filtration. For use as culture medium, sterile stock solutions were diluted with sterile deionized water and mixed with serum. *Subculture of cells* was accomplished by a) washing cultures with 2-3 changes of NaCl-KCl solution; b) detaching cells from glass by incubation with 0.5% Difco "certified" 1:125 trypsin in NaCl-KCl solution for 5 minutes at 37°C; c) harvesting cells by centrifugation and resuspending them in NaCl-KCl solution for counting with a hemocytometer, and diluting to desired density; and d) inoculating cells into growth medium warmed to 37°C and equilibrated to pH 7.2-7.4. Cultures were fed every third day by complete medium change; acid production was countered by appropriate addition of bicarbonate to maintain pH. *Measurement of culture* growth was compared for a) hemocytometer counts of cells dispersed with 0.5% trypsin, collected by centrifugation, and diluted to required population densities; b) dry weight estimation (4 x 10^7 EE cells weighed 0.7 $\pm$ 0.2 mg); c) turbidity of dispersed cell suspensions (Fig. 1); and d) assay of cell nitrogen by Koch-McMeekin Nessler method and use of Klett-Summerson photoelectric colorimeter with No. 54 filter. Dry weight was an insensitive and inaccurate measure; turbidity was measurable accurately but re-

FIG. 1. Turbidity of human EE (Minn. 55-12-1) cell suspensions, as read in a Klett-Summerson photoelectric colorimeter, filter 42. Cell suspensions were prepared from 10-day cultures.

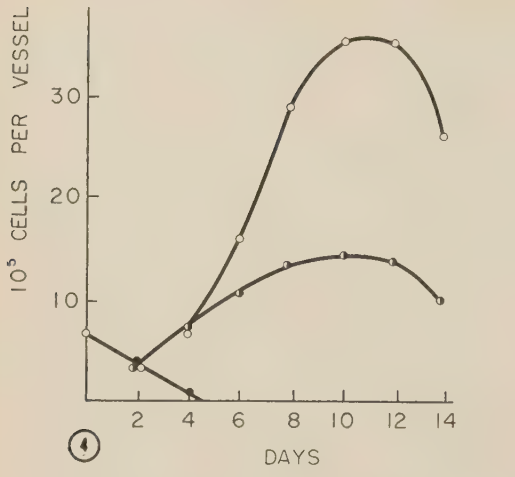
FIG. 2. Total nitrogen content of human EE (Minn. 55-12-1) cells, determined by the Koch-McMeekin Nessler method.

FIG. 3. Growth of human EE (Minn. 55-12-1) cells at 37°C in medium containing 10% whole human serum, 0.1% yeast extract and 12 mM glucose, measured by total cell count (half-solid circle) and nitrogen determination (open circle).

quired too many cells. Cellular nitrogen was directly proportional to cell count over a wide range (Fig. 2). Growth curves based on cell count and nitrogen measurement were compared for EE cells grown in standard medium with glucose (Fig. 3). During days 1 and 2 of culture, a drop in cell number was not accompanied by a reduction in cellular nitrogen. Toward the end of the growth cycle on days 10-12 nitrogen continued to rise although cell count leveled, and on days 12-14 cellular nitrogen dropped more rapidly than cell count. These discrepancies were compatible with assumption of differences in associated rates of protoplasm synthesis and cellular division(5). Since the present study was concerned with qualitative capacity of individual carbohydrate media to support production of new cells rather than with quantitative capacity to apply derived energy or metabolites to protein synthesis, cell count was adopted as a sufficient measure of culture growth. *Viable inoculum* for dispersed plane cultures was measured as efficiency-of-cell establishment (EOE), *i.e.*, the fraction of the cells in the inoculum that attached to the glass. EOE was measured by hemocytometer counts of a) cells and cell ghosts floating in medium 18 hours after culture inoculation, or b) cells of washed monolayers completely dispersed by trypsin. Agreement of the two measures is illustrated by a typical experiment where 3.6 x 10^5 cells and ghosts were counted in medium taken from cultures inoculated with 6 x 10^5 cells: 1.8 x 10^5 cells were recovered after removal of medium and detachment of cells from glass. *Glucose content* of media was determined as a) reducing sugar measured by Folin-Wu method and colorimetric assay with Klett-Summerson photoelectric colorimeter (filter No. 42); and b) fermentable substrate measured manometrically by use of *Candida stellatoidea*(6). Eighteen-hour yeast cultures were harvested by centrifugation, washed 4X with cold deionized water, depleted of fermentable reserves by incubation for 4 hours at 20°C in 0.06 M phosphate buffer at pH 4.5, and added to Warburg vessels in 2 ml of phosphate buffer to a density of 1.5 x 10^8 yeasts/ml. Side arms were loaded with test medium diluted appropriately in phosphate buffer. Manometric measurement of carbon dioxide was continued until evolution was less than 1 μl/15 minutes by 2 consecutive readings.

Results. Glucose depletion of serum-yeast-extract growth medium was accomplished by microbiological treatment. Dialysis was avoided since it might deplete growth factors present in the complex basal medium. *Candida stellatoidea* from 18-hour broth cultures was washed twice with cold deionized water (7) and added to growth medium to a concentration of 5 x 10^7 yeasts/ml. Seeded medium was incubated stationary at 30°C for 4-6 hours before the yeasts were removed by centrifugation. Growth of EE cells in treated medium with and without added glucose indicated that yeast treatment had depleted glucose satisfactorily, without apparent production of toxic material (Fig. 4). By colorimetric reducing-sugar assay, untreated medium contained 186 $\pm$ 38 mg glucose/liter, and treated medium 22 $\pm$ mg/liter (88% depletion). These media had been prepared without added glucose: standard medium with 12 mM added glucose contained 2,328 $\pm$ 294 mg glucose/liter, in close agreement with the expected value. By manometric assay, untreated no-sugar medium contained 154 mg glucose/liter (42.1 $\pm$ 4.3 μl CO_2 evolved from 1 ml), treated no-sugar medium contained 31 mg glucose/liter (8.4 $\pm$ 1.3 μl CO_2 from 1 ml), and standard 12 mM glucose medium contained 2,206 mg/liter. Results obtained by chemical and microbiological assays thus were similar. *C. stellatoidea* grown in glucose broth fermented fructose and glucose, but not lactose, sucrose, d+maltose, d+galactose, mannitol, cellobiose, d+xylose, l+arabinose, d–melibiose, d+melezitose, d+trehalose, d+turanose, d+raffinose, sodium lactate, sodium pyruvate, ethanol or sodium acetate. Representative values of carbon dioxide evolution are shown in Table I. By these results, *C. stellatoidea* fermentation was sufficiently selective to be a reliable method for glucose depletion of cell culture media.

Toxicity of sugars for EE cells was tested by adding sucrose, maltose, galactose, lactose, arabinose, xylose, fructose, melibiose, melezitose, trehalose, raffinose, or mannose at con-



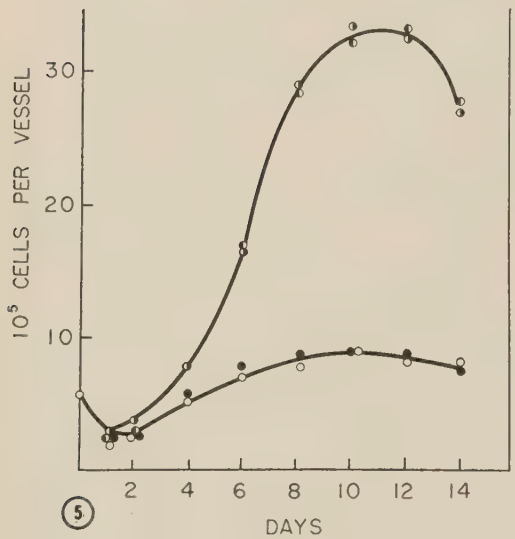


FIG. 4. Growth of human EE cells at 37°C in medium containing 10% whole human serum and 0.1% yeast extract, and depleted of glucose by treatment with *Candida stellatoidea*. The 3 curves depict growth obtained with no added glucose (solid circle), with 1 mM added glucose (half-solid circle) and with 12 mM added glucose (open circle).

FIG. 5. Growth of human EE (Minn. 55-12-1) cells in yeast-treated medium containing 10% whole serum, 0.1% yeast extract and glucose or fructose, as measured by hemocytometer counts. The 4 curves show growth with 12 mM glucose (circle, solid right), 1 mM glucose (solid circle), 12 mM fructose (circle, solid left) and 1 mM fructose (open circle).

centrations of 1.08, 2.16 or 4.32 g/liter to glucose medium. None of the carbohydrates prevented growth of EE cells or affected morphology at these concentrations.

Growth in carbohydrate media was measured for an inoculum of 6 x 10^5 EE cells in culture bottles containing 5 ml of carbohydrate medium pretreated by yeast fermentation. In *turanose, melezitose, melibiose* or *arabinose* medium, only about 10% of inoculated EE cells were attached to glass after one day of incubation, and these were detached by the fourth day. In *lactose* medium, 15% of cells attached themselves to the glass and flattened as if to initiate growth, but then detached rapidly as if some toxic product had been formed. In *raffinose* and *trehalose* media the cells adhered to the glass and were maintained for several days but without significant growth. In other experiments, EE cultures established on glass in glucose medium were washed repeatedly with NaCl-KCl solution after 3 days of incubation, and overlaid with yeast-treated sugar medium. After 8 days no cells survived in medium containing lactose, turanose, melezitose, melibiose or arabinose. Raffinose and trehalose maintained the cells but did not support multiplication. Cultures started in fructose or glucose medium exhibited identical EOE of 30%, and similar growth curves (Fig. 5). Although EE cells grew well in yeast-treated maltose medium, this glucose disaccharide was not studied further because manometric assay of glucose in yeast-treated maltose medium showed an increase from an initial concentration of 50 mg glucose/liter to about 750 mg/liter after 12 hours incubation at 37°C. Under these conditions growth permitted by maltose could not be differentiated from that permitted by glucose possibly released by serum maltase. In initial experiments, sucrose medium maintained EE cells for several days

TABLE I. Ability of *Candida stellatoidea* to Ferment Carbohydrates.

Substrate	CO_2 evolved, μl/hr	Substrate	CO_2 evolved, μl/hr
glucose	550	melibiose	19
fructose	465	cellobiose	18
galactose	262 (1st hr)	melezitose	9
	110 (2nd ")	sucrose	18
	43 (3rd ")	trehalose	9
	0 (4th ")	mannitol	12
maltose	35	raffinose	19
arabinose	10	turanose	4
lactose	29	buffer	8
xylose	23		

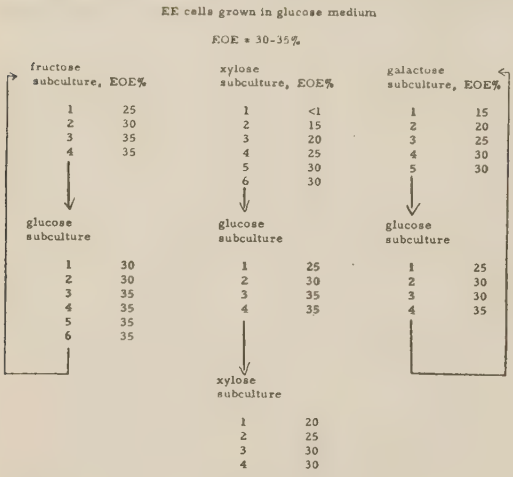
with little or no growth. Occasionally 20% of the inoculum survived and almost immediately multiplied. This irregular behavior suggested a) adaptive enzyme formation or b) uncontrolled variation of culture medium, rather than selection of pre-existing capable cells. Ability of sucrose to permit multiplication of EE cells was dependent on the serum pool employed. To detect any serum invertase, yeast-treated sucrose medium with and without added EE cells was assayed for reducing sugar by the Folin-Wu method. Although yeast-treated sucrose medium alone showed no increased content of reducing sugar after 4 days of incubation, medium with added EE cells rapidly accumulated reducing sugar. In one experiment, reducing sugar concentration increased from zero-time level of 28 mg/liter to 474 mg/liter after 12 hours, and to 840 mg/liter after 24 hours; during the following 48 hours reducing sugar concentration varied from 600 to 800 mg/liter. Results by manometric determination of fermentable substrate were similar. Since neither EE cells nor serum alone was able to convert sucrose to reducing sugar, it appeared that EE cells interacted with certain serum pools to manifest invertase activity. The cells may have activated a serum invertase precursor or deactivated an inhibitor. With the above described carbohydrates then, EE cells were a) unable to use the carbohydrate during growth, b) able to use the unaltered carbohydrate during growth or c) able to grow in medium containing products produced from the presented carbohydrate by the serum or the serum and cells together.

Selective growth in carbohydrate media was observed with 2 sugars. Commercially available *galactose* was freed of up to 2% contaminating glucose by yeast-treatment. EE cells transferred from glucose medium to galactose medium established themselves with 15-20% efficiency, compared to 30-35% EOE for glucose-to-glucose subculture. Cells transferred to galactose medium initially were smaller and grew slower than cells in glucose medium. Equivalent cell populations in the same time period frequently lowered the pH of glucose medium to 6.5, but rarely lowered the pH of galactose medium below 7.0. On second transfer in galactose medium, cells attained more nearly the size of cells in glucose medium; again the pH of galactose medium was reduced less than that of glucose medium by cell multiplication. By the fourth subculture in galactose medium, cells were as large as glucose-grown cells and their EOE had increased to 30-35%. *Xylose* medium was yeast-treated for uniformity, although commercial xylose contained very little contaminating glucose. Only 7 of 46 EE *cultures* survived transfer from glucose to xylose medium, and these grew out from a small number of cells (EOE $<1\%$). Efficiency of establishment increased to 10% with the second xylose subculture and progressively to 35% by fourth subculture. After several transfers in xylose medium, cells multiplied as rapidly as in glucose medium; again, minimal lowering of xylose-medium pH was seen. With galactose and xylose, then, ability to grow in the carbohydrate medium could be acquired by EE cultures.

Heritability of power to multiply in particular carbohydrate media was tested by serial subculture. Cells transferred from glucose medium to fructose medium exhibited an EOE of 25-30% and grew immediately. Continued fructose-to-fructose subculture raised the EOE to 30-35%. About 30% of fructose-grown cells survived transfer to glucose medium and multiplied. EOE and growth rate were not affected greatly by retransfer of these glucose-grown cells to fructose medium (Table II). Growth curves were not significantly different from those shown in Fig. 5. Therefore ability to multiply in fructose medium was hereditary and constitutive; that is, the ability was transmitted through many cellular generations in the absence of substrate.

Only small numbers of parental-culture EE cells grew on transfer from glucose to xylose medium: these achieved a 30% EOE after 4-6 subcultures representing 20 to 40 cell generations in xylose medium. The maximum efficiency corresponded to that of glucose-to-glucose subculture of control cells. Xylose-grown cells established themselves with efficiency of 25-30% on transfer to glucose medium, and with 20% efficiency on retransfer to xylose medium (Table II). Thus the altered capacity of EE populations to grow in

TABLE II. Hereditability of Capacity of Human Esophageal Epithelial Cells (Minn. 55-12-1) to Grow in Particular Carbohydrate Media.



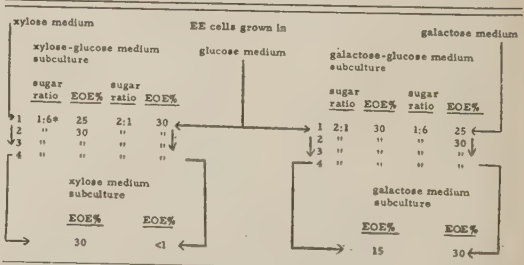
xylose medium, once obtained by selection in xylose, was hereditary and constitutive.

Results for galactose were less decisive but clearly different from those for xylose. As with xylose medium, EE populations propagated by galactose-to-galactose transfer exhibited an EOE of 30%, like that of control populations propagated by glucose-to-glucose transfer (Table II). Galactose-grown cells were transferred readily to glucose medium (EOE = 25%), but were retransferred less easily to galactose medium after limited growth in glucose medium (EOE = 15%). It appeared that the increased ability of galactose-grown cells, contrasted to glucose-grown cells, to initiate growth in galactose was adaptive; that is, the capacity to grow in galactose medium was transmitted from culture to subculture in the absence of galactose but was quantitatively dependent on presence of galactose. Since full efficiency of culture capacity to grow with galactose was not achieved with one passage of glucose-grown cells in galactose medium (Table II), it is not clear whether adaptive enzyme production was the sole mechanism operating.

Similar experiments were performed with growth media containing mixed sugars. Grams-per-liter ratios of glucose:xylose or glucose:galactose of 1:2 and 6:1 were used. Ability of glucose-grown EE cells to initiate growth in xylose or galactose medium was not increased by prior subculture in 12 mM glucose medium, supplemented with xylose or galactose respectively. Conversely, once the cells had achieved increased EOE during passage in xylose or galactose medium, this capacity was maintained during passage in medium containing 6 times as much glucose as xylose or galactose (Table III). Supplementation of glucose medium with xylose conferred no selective advantage on xylose-capable cells in glucose-grown populations, and glucose reinforcement of xylose medium conferred no selective advantage on any xylose-incapable cells in xylose-grown populations. Subculture in glucose-fortified galactose medium did not reduce efficiency of galactose-grown cells for establishment in galactose medium, nor did subculture in glucose-fortified galactose medium increase efficiency of glucose-grown cells for establishment in galactose medium.

Discussion. Capacity of human cells in continuous culture (Minn. EE 55-12-1 strain) to multiply in various carbohydrate media was studied with parental rather than clonal populations, to observe the widest range of behavior. The parental EE stock was able to grow in media containing glucose and fructose whether cultivated previously in glucose or in fructose medium. Lack of similar capability for use of sucrose and lactose contrasts with ability of the human animal. *In vivo,* sucrose and lactose capability may result from cellular interaction or from adaptive production of invertase and lactase under peculiar inductive conditions. Xylose capability of xylose-

TABLE III. Effect of Subculture in Carbohydrate Mixtures on Capacity of Esophageal Epithelial Cells (Minn. 55-12-1) to Grow in Carbohydrate Media.



* g/liter ratio of sugars as indicated by subheading.

grown EE cells was not dependent on presence of xylose but was limited to a few cells in glucose-grown parental populations. Experiments presented here did not establish whether the selected xylose-capable cells were mutants or progeny of cells existing in the tissue source of the EE strain. Galactose capability by contrast appeared adaptive. Results of experiments with mixed sugars indicated that xylose and galactose capabilities were alternative rather than substitutive to glucose capability. Given interpretations of data are subject to the reservation that analyses were based on establishment efficiencies of less than 50%. It is not yet known what additional selective forces thus were imposed by conditions of experiment. Before undertaking determinative studies of representative numbers of clones separated from the parental population, it appears desirable to investigate possible effects of very small amounts of one sugar on capacity of cells to make primary use of another sugar for growth. The necessarily complex media used herein for propagation, as noted, were highly depleted but not totally freed of glucose.

Summary. Human esophageal epithelial cells (Minn. 55-12-1 strain) in serum medium microbiologically depleted of glucose were unable to grow with lactose, raffinose, trehalose, turanose, melezitose, melibiose or arabinose as sole carbon sources. Cultures in depleted medium grew well with maltose and occasionally with sucrose by production of reducing sugar through serum or cell-serum activity. Ability to grow in glucose-depleted medium containing fructose, xylose, and galactose was inherited. Acquired xylose capability of cultures appeared to result from selective enrichment of populations with constitutively xylose-capable cells. Expression of capability of cultures to grow in galactose medium was modified reversibly by prior exposure to galactose or glucose.

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Dissociation of Some Injected Antigen-Antibody Complexes *in vivo.* (25465)

HARRY WALTER AND HERBERT ZIPPER (Introduced by Felix Haurowitz)
Radioisotope Service, Vet. Admin. Hospital, Brooklyn, N. Y.

As a natural outgrowth of many studies (1-9) exploring the fate of antigens in normal, x-rayed or sensitized animals, recent investigations(10-12) have probed certain aspects of biological activity or metabolism of antigen-antibody complexes directly. Antigen-antibody (Ag-Ab) complexes prepared *in vitro* using radio-iodinated antigen or antibody(10, 11) were injected intravenously into rabbits and elimination from serum was traced as was their initial deposition and subsequent catabolism in various tissues(11). We previously found(11) that clearance from blood of intravenously injected, insoluble, suspended Ag-Ab complexes as indicated by trace labeled antigen proceeds more rapidly in sensitized than in control animals. It seemed to us that the circulating antibody in sensitized rabbits reduced the dissociation of injected complexes thus expediting their removal from the circulation. We also showed that in normal animals antigen-antibody complexes do, at least to some extent, dissociate. We present here further evidence for (a) dissociation of antigen-antibody complexes in sera of rabbits (normal and sensitized) *in vivo* and (b) rapid

equilibration of the labeled Ab moiety of the injected complex with the circulating Ab. This was demonstrated by comparing elimination from serum of Ab-labeled Ag-Ab complexes to elimination of Ag-labeled Ag-Ab complexes(11).

Methods. Rabbits were immunized by repeated(6-8) subcutaneous injections of 60 mg/animal of beef gamma globulin at 3-4 day intervals. Eight to 10 days after final injection, rabbits showing qualitatively similar titers were exsanguinated by heart puncture, the blood allowed to clot and the serum collected. Preparation of beef gamma globulin (BGG) trace labeled with I^{131} (I*BGG), preparation of insoluble antigen-antibody complexes between I*BGG and anti BGG from the gamma globulin fraction of rabbits sensitized to BGG, and subsequent suspension in saline and intravenous injection of these complexes into normal or BGG sensitized rabbits have been described(11). The gamma globulin fraction of rabbits immunized against BGG was prepared(11) and iodinated by the method of Warren and Dixon(13). In a typical preparation approximately 550 mg of rabbit gamma globulin and 40 mc of I^{131} containing 5.6 mg of potassium iodide as carrier were used. The iodinated rabbit gamma globulin was immediately precipitated by addition of equal volume of neutralized saturated ammonium sulfate, centrifuged down, dissolved in a few ml of water and extensively dialyzed against saline in the cold. Although we found that this procedure gives only 3.7% of the theoretical yield in iodination of the protein (necessitating high activity used in the experiment), the titer of the iodinated antibody appears essentially unaffected. Complexes between unlabeled BGG and I* anti BGG were prepared in the manner described for labeled BGG and unlabeled anti BGG(11). Ag-Ab complexes in which the BGG is labeled are designated as I*BGG-anti BGG while those in which the antibody is labeled are designated as BGG-I* anti BGG. Rabbits sensitized to BGG or normal rabbits were intravenously injected(11) with 3.3 mg containing 2.6 x 10^6 cpm of BGG-I* anti BGG. Rabbits were bled periodically from ear marginal vein, blood was allowed to clot, and serum removed. Dry protein powders were prepared for counting(14) and the total sample weighed and counted in a scintillation well-counter.

Results of these experiments, and of those to which we wish to compare them(11), are presented in Fig. 1. We express results as relative specific activities (RSA) which have previously been defined(14) as:

$$\text{RSA} = \frac{\text{counts/min. in 100 mg protein}}{\text{counts/min. inj./g body wt}}.$$

RSA values allow direct comparison of data obtained from animals of the same species but of different weight.

Fig. 1 shows protein-bound serum activities obtained over 7 days when sensitized or normal rabbits are injected with BGG-I* anti BGG complex or with I*BGG-anti BGG complex intravenously *via* the ear marginal vein. Each point in Fig. 1 represents the average RSA value obtained from 4 similarly injected rabbits.

It can be seen that labeled antibody admin-

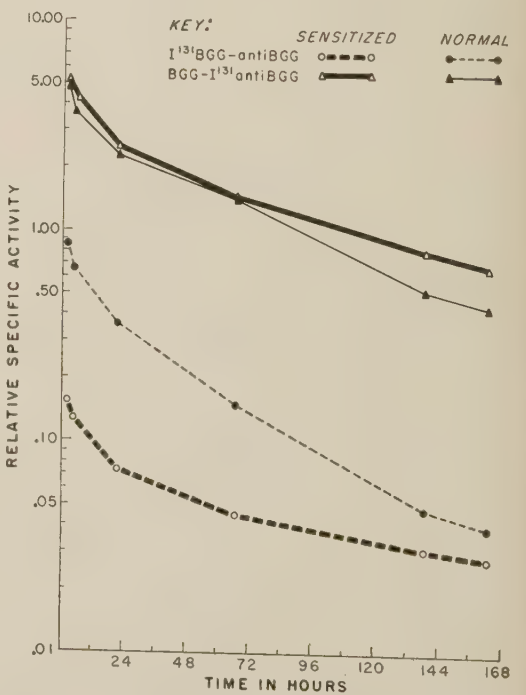

FIG. 1. Protein-bound relative specific activity of rabbit sera at various times after intrav. inj. with BGG-I^{131} anti BGG complex or with I^{131} BGG-anti BGG complex.

istered as part of an antigen-antibody complex is removed from the circulation of normal controls and rabbits sensitized to BGG at quite similar rates. On the other hand, when elimination of antigen-antibody complexes is traced by tagging the antigen moiety(11) (Fig. 1), disappearance of the label is much more rapid and, furthermore, is considerably influenced by presence or absence of circulating antibody in blood. It thus appears that, in blood circulation *in vivo,* there is dissociation of the injected complex and equilibration between labeled antibody administered in the complex and antibody in the circulation of sensitized animals. The small amount of labeled antibody in the complex must *rapidly* equilibrate with the large amount of antibody in the circulation since no difference is apparent in rate of clearance of the labeled antibody administered in the complex in sensitized and normal animals. In experiments in which the antigen component of the complex is labeled, excess circulating antibody assures that the label (Ag) will continue to form part of an Ag-Ab complex and be, therefore, more rapidly removed from the circulation.

The difference in levels of activity of labeled Ag and of labeled Ab in Ag-Ab-injected *normal* rabbits, apparent shortly after injection, is probably due to the initial clearance of injected Ag-Ab complex. Even complexes of Ab:Ag ratios smaller than those injected (*i.e.* partially dissociated complexes) are still very rapidly removed from the circulation.

Summary. When rabbits are injected with antigen-antibody complexes in which the antibody moiety is labeled with I^{131}, no difference is found in its clearance from blood between animals sensitized to the antigen and normal controls. This finding is quite different from previous results when similar complexes with the antigen moiety labeled are injected(11). In the latter case radioactivity is cleared much more rapidly in sensitized rabbits as compared to their controls. The dissociation of antigen-antibody complexes *in vivo* and the rapid equilibration of the labeled Ab of the injected complex with the Ab circulating in sera of sensitized rabbits are given as explanations for these findings.

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Pyridine Nucleotide-Cytochrome C Reductases in Rats Exposed to Low Oxygen Tensions. (25466)

Baltazar Reynafarje and James Green (Introduced by Alberto Hurtado)
Inst. of Andean Biology and Dept. of Pathological Physiology, Faculty of Medicine, Lima, Perú, and School of Aviation Medicine, Dept. of Physiology, Randolph Air Force Base, Randolph Field, Texas

It has been shown that tissues from high altitude guinea pigs have a significantly higher succinoxidase system activity than those from sea level(1). However, "Antimycin titer" and cytochrome oxidase are little changed with altitude and the latter only in some tissues, indicating that the process of adaptation to high altitude may not necessarily involve modifications in all steps of a multienzyme system but rather in those which are rate-limiting factors for that system. DPNH and TPNH-cytochrome c reductases have been pointed out as very important steps in the respiratory chain, since the flow of electrons between, and through these 2 elements of the chain may be modified by a specific transhydrogenase in keeping with metabolic and energetic needs of the animal(2). All these facts prompted us to undertake a preliminary study concerning DPNH- and TPNH-cytochrome c reductases in liver of rats exposed to low oxygen tensions and compare the results with those obtained in sea level rats.

Methods. Two series of adult male albino rats were selected. Both groups were kept under the same standard conditions, except that rats for high altitude series were placed in low pressure chambers at a simulated altitude of 18,000 feet, for 23 hours daily, during 6 months. After killing animals by decapitation the liver was removed and a 10% homogenate was prepared in cold isotonic sucrose (0.25M). Cell fractions were obtained following the method of Schneider and Hogeboom(3). Enzymatic reduction of cytochrome c after addition of one of the reduced pyridine nucleotides was followed at 550 mμ in a model D. K. No. 2 Beckman Recording Spectrophotometer, at room temperature. The reaction mixtures and assay procedures were the same as those described previously(4). Specific activity is given as μM of cytochrome c reduced/minute/g of protein. The absorbancy index used for this reduction was 1.91×10^4 1 $mole^{-1}$ cm^{-1} (absorbancy index of reduced cytochrome c at 550 mμ-absorbancy index of oxidized cytochrome c at 550 mμ)(5).

Results. The liver from animals exposed to low oxygen tensions shows a higher DPNH-cytochrome c reductase activity than that from animals staying at sea level (Table I). Percentage of increment in activity of this enzyme is nearly the same in whole homogenate and in cell fractions. On the other hand, TPNH-cytochrome c reductase does not show any increment in animals exposed to high altitude; on the contrary, it tends to be lower than in the sea level group, although the difference has no statistical significance. To understand the results better, disregarding individual variations, ratios between DPNH- and TPNH-cytochrome c reductases were calculated for each animal, thus eliminating any variability due to changes in room temperature from one experimental day to another. Table II shows relative activities of these en-

TABLE I. Specific Activities of Pyridine Nucleotide-Cytochrome C Reductases in Liver from Sea Level and High Altitude Acclimated Rats.

	DPNH-cytochrome c reductase			TPNH-cytochrome c reductase		
	Sea level	High altitude		Sea level	High altitude	
	Means ± S.E. (8)*		P	Means ± S.E. (7)*		P
Whole homogenate	332.5 ± 22.5	463.1 ± 24.0	<.01	17.1 ± 5.4	14.6 ± 3.2	>1
Mitochondria	456.3 ± 29.3	717.8 ± 32.0	"	32.2 ± 6.4	29.4 ± 4.2	"
Microsomes	677.6 ± 67.4	1076.2 ± 85.6	"	26.3 ± 5.0	19.2 ± 3.7	"

* No. of animals. P = Probability based on t test of significance of differences between means.

TABLE II. Relative Activities of Cytochrome C Reductases in Rat Liver and Its Cell Fractions during Acclimatization to Simulated High Altitudes.

Tissue sample	Altitude	Cytochrome c reductases DPNH-	TPNH-
Whole homogenate	Sea level	19.4*	1
	18,000 ft	31.7	1
Mitochondria	Sea level	14.2	1
	18,000 ft	24.4	1
Microsomes	Sea level	25.7	1
	18,000 ft	56.0	1

* Figures are avg from 7 to 8 individual ratios.

zymes for each group of animals. In the altitude series the activity of the DPNH-cytochrome c reductase, with respect to the TPNH-system, is about twice as much as in the sea level control. It seems therefore, that during the process of adaptation to a low pressure environment there is a selective modification in transport of electrons toward oxygen. Both, DPNH- and TPNH-systems use oxygen as a terminal acceptor for electrons, though it is apparent that only the former generates high-energy phosphate bonds(2). These findings show an increase in activity of the high-energy generating system indicating that one of the features of acclimatization to a low oxygen tension atmosphere could be the improvement of those mechanisms involved in furnishing utilizable energy.

Summary. Pyridine nucleotide-cytochrome c reductase activities of whole rat liver and its cell fractions were determined in high altitude exposed rats and the results compared with those obtained in sea level rats. DPNH-cytochrome c reductase activity was higher in the low oxygen tension exposed rats, whereas the TPNH-cytochrome c reductase activity did not show any appreciable change. The significance of these findings is discussed.

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Pyridine Nucleotide-Cytochrome C Reductases in Acclimatization to Cold. (25467)

BALTAZAR REYNAFARJE AND ROBERT CHAFFEE (Introduced by Alberto Hurtado)
Inst. of Andean Biology and Dept. of Pathological Physiology, Faculty of Medicine, Lima, Perú, and School of Aviation Medicine, Dept. of Physiology, Randolph Air Force Base, Randolph Field, Texas

Liver mitochondria from cold adapted hamsters have a higher succinoxidase and glutamic acid oxidase systems activity than those from control animals. Furthermore the succinic dehydrogenase and cytochrome oxidase activities are elevated in the former. To understand better the extent of enzymatic changes taking place during this adaptation, studies involving DPNH* and TPNH-cytochrome c reductases were undertaken.

Material and methods. Normal adult male hamsters kept in the cold (3° ± 2°C) for 70 days were killed by decapitation and their livers removed and placed in ice-cold isotonic sucrose. Mitochondria were prepared from a 0.25 M sucrose homogenate following the classic technic of Schneider and Hogeboom (1). Microsomes were obtained by centrifuging the mitochondrial supernatant at 105,000 x g for 50 minutes in Spinco preparative ultracentrifuge. Reduction of cytochrome c upon addition of one of the reduced pyridine nucleotides was recorded at 550 mμ in a model DK No. 2 Beckman spectrophotometer. Rates of reaction were measured at room tem-

* Abbreviations used: DPNH and TPNH = reduced diphospho and triphospopyridine nucleotides.

TABLE I. Specific Activities of Pyridine Nucleotide-Cytochrome C Reductases in Liver-Cell Fractions from Control and Cold Exposed Hamsters.

		DPNH-cytochrome c reductase		TPNH-cytochrome c reductase	
		Mean ± S.E. (15)*	P	Mean ± S.E. (7)*	P
Mitochondria	Control	429.7 ± 29.4	>1	45.1 ± 4.0	>1
	Cold	466.5 ± 33.2		65.7 ±14.1	
Microsomes	Control	897.1 ± 54.8	< .02	15.3 ± 1.2	< .001
	Cold	1359.2 ±167.6		26.9 ± 2.3	

* No. of animals. P = Probability based on t test of significance of differences between means.

perature, and always one control and one cold exposed animal were studied/experiment. Standard reaction mixtures and assay procedures have been described(2). Specific activity of samples was expressed in terms of μM of cytochrome c reduced/minute/g of protein at 550 mμ. Protein determinations were carried out by biuret reaction.

Results are presented in Table I. It may be noticed that the enzymatic activity of DPNH- and TPNH-cytochrome c reductases is not significantly changed in the mitochondrial liver fraction from cold exposed hamsters as compared with controls. Although the mean value of the TPNH-cytochrome c reductase is higher in the cold adapted animal, the difference has no statistical significance due to large individual variations. In the microsomal fraction, on the other hand, the activity of these enzymes is frankly elevated in the cold exposed group of animals. The data show an increment in activity of TPNH-cytochrome c reductase of about 77% over controls.

Discussion. It is of interest to consider the possible implications of these findings in the mechanism of cold acclimatization. Kaplan *et al.* showed that oxidation of TPNH is carried out with less generation of energy-rich phosphate bonds than the oxidation of DPNH (3). In turn, Vignais advanced the idea that oxidation of TPNH is accomplished with more heat evolution than oxidation of DPNH and that the increase in B.M.R. in the rat during Vit. A deprivation could be due to increment in oxidation of TPNH(4). Scholander *et al.*, on the other hand, have shown that in men acclimatized to cold, heat production remained much higher than the basal rate before acclimatization and that this increment is attained with no extra oxygen consumption(5). It is evident from these findings that the mechanism of cold acclimatization involves a metabolic adjustment resulting in increased heat production. It is also clear that oxidation of TPNH, as judged from this report, plays an important role in this acclimatization. Furthermore, the increment in activity of these reductases which occurs only in microsomes, and not in mitochondria which are regarded as the oxidative units in the cell, may signify that utilization of oxygen is not involved in the process as has been demonstrated in men by Scholander(5).

Summary. A study of the pyridine nucleotide-cytochrome c reductases activity was carried out in liver cell fractions (mitochondria and microsomes) from cold exposed hamsters. Control animals were studied simultaneously. Both DPNH- and TPNH-cytochrome c reductases had a higher activity in microsomes of cold adapted hamsters than in those of controls; the latter nevertheless increases more conspicuously. In the mitochondria, in turn, these 2 systems did not show significant changes. The possible implication of these findings in cold adaptation is discussed.

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Lysis by Human Oral Bacteria of Collagen Altered By Ethylene Oxide. (25468)

Stephan E. Mergenhagen, David B. Scott and Henry W. Scherp
(Introduced by Isadore Zipkin)
National Inst. of Dental Research, N.I.H., P.H.S., U. S. Dept. of H.E.W., Bethesda, Md.

Sterilization with ethylene oxide increased digestibility of collagen in tissue by broth cultures inoculated with human gingival accumulations(1,2,3). Failure of digestion by several pure cultures isolated from such mixtures indicated a synergistic mechanism of collagenolysis(1). Similarly, collagen fibrils reconstituted from acid-soluble collagen of rabbit skin were digested by such mixed cultures but not by pure isolates from them, either alone or in combination(4). However, after treatment with ethylene oxide the reconstituted collagen was digested moderately by a few of these same pure cultures and extensively by their combination. Concomitant morphological alterations of collagen fibrils were followed by electron microscopy.

Materials and methods. The test for collagenolysis was similar to that used previously (4). Two-ml aliquots of a neutral 0.2-0.5% solution of acid-soluble collagen were gelled at 37°C and exposed to 11% ethylene oxide gas in a desiccator for 16 hours. Eight ml of a special hydroxyproline-free broth were added and inoculated with 0.1 ml portions of 48-hour broth cultures of the respective pure cultures. The tubes were then incubated for 7 days at 37°C under 95% nitrogen and 5% CO_2. Following centrifugation, aliquots of sediments and supernatants were analyzed for hydroxyproline after acid hydrolysis. Since the oral microbiota readily catabolizes the hydroxyproline-containing products of collagenolysis, the loss of total hydroxyproline served as an indicator of extent of collagenolysis(4). Five strains of Gram-negative fusiform bacilli (F1, F3, F4, F5, F7) were isolated from human saliva on a selective medium(5) and transferred to fluid thioglycollate medium (Difco) containing 0.5% calcium carbonate. Five strains (V1, V2, V3, V4, V5) of Gram-negative anaerobic cocci (genus *Veillonella*) were isolated from human saliva on a selective medium(6) and maintained in the same medium lacking agar, vancomycin, and basic fuchsin. Two strains of diphtheroid bacilli (PC7, PC8) and 3 strains of viridans streptococci (PC1, PC2, PC6) were isolated on sheep-blood agar from cultures of mixed human gingival scrapings in a casein hydrolyzate broth(4) and subcultured in this broth with addition of 0.5% glucose. Specimens of the reconstituted collagen were prepared for electron microscopy in the form of dried microdrops of suspensions and as thin sections of gel. For the former, small quantities of gel were rendered salt free by repeated suspension in triple-distilled water and centrifugation. Microdrops of final suspensions were placed on electron microscope screens covered with carbon substrates, allowed to dry in air, and shadowed with palladium. Gel samples to be sectioned were fixed for 2 hours in a buffered osmium tetroxide-potassium dichromate mixture, dehydrated in ethanol, and embedded in a mixture of butyl and methyl methacrylates. Sections were cut with a Porter-Blum microtome equipped with glass knives. To afford closer study of the fibrils, the embedding medium was removed from some of the sections, which were then shadowed with palladium.

Results. Marked alterations in morphological characteristics of the gels after ethylene oxide treatment were observed under the electron microscope in both microdrop and sectioned preparations. Before treatment the gels consisted almost entirely of a meshwork of typical collagen fibrils with clearly defined 640A cross-striations (Figs. 1 and 4). After long exposures (16 hours) to ethylene oxide only a few of these fibrils could be found, and the gel seemed instead to be made up of much finer unstriated fibrils, arranged either as a loose network (Fig. 2) or as a compact mass (Fig. 3). Concomitantly, the collagen tended to pass from gel to sol state. Since, however, all hydroxyproline-containing material remained completely precipitable by 2.5% tan-

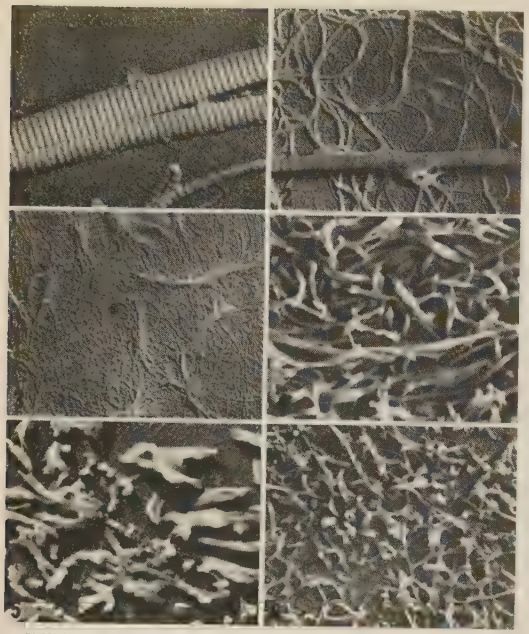

FIG. 1-3. Electron micrographs of microdrop suspensions of heat-gelled acid-soluble collagen before (Fig. 1) and after (Fig. 2, 3) exposure to 11% ethylene oxide gas for 16 hr. × 15,000.

FIG. 4-6. Electron micrographs of sections of heat-gelled acid soluble collagen before (Fig. 4) and after exposure to 0.1% aqueous ethylene oxide for 1 hr (Fig. 5) and 3 hr (Fig. 6). × 11,500.

nic acid, the collagen molecule itself was presumably not split. When a series of specimens exposed for shorter periods was examined to determine the way in which the collagen fibrils disintegrate, an impression was gained that they first become somewhat swollen, then break up into short lengths and finally decompose completely into the fine fibrillar units. Pronounced alterations were observed after as little time as one hour (Fig. 5) and by the end of 3 hours most fibrils completely lost their identity as collagen (Fig. 6). Fibrillar swelling similar to that seen in Fig. 5 was reported in histological sections of human gingival tissue after exposure to ethylene oxide vapor(2).

Table I presents the essential results of a typical experiment demonstrating that these altered collagen fibrils became slightly susceptible to digestion by certain pure cultures of oral bacteria, but most significant was the observation that in combination these cultures effected a 77% reduction in the collagen present as compared with a 59% reduction by *Clostridium histolyticum*. In repeated tests these cultures and their combination failed completely to attack untreated reconstituted collagen(4). These results substantiate previous suggestions of a synergistic mechanism of bacterial lysis of altered collagen(1,3). However, since growth of such organisms as the fusiform bacilli was generally greatly enhanced in the combined cultures, the increased digestion might have resulted at least in part from symbiotic stimulation of growth and metabolism. Finally, it is certainly possible that other combinations of oral bacteria might effect similar reactions.

The microscopic changes observed in these altered collagen fibrils recall the electron microscopic demonstration that collagen fibrils, in areas of rabbit skin where inflammation had been induced by the Arthus phenomenon, exhibited an absence of normal periodic cross striations, variations in density, and irregularities of the margins with evidence of swelling and fragmentation(7). Similarly, the increased susceptibility to bacterial degradation of the collagen fibrils shortened by ethylene oxide (Fig. 6) recalls the report(8) that when native tendon was cut into small pieces (less than 1 mm), the fibers became digestible by trypsin, indicating that they were digested primarily at the cut ends (*cf.* also (9)). In other words, finely divided collagen should be more readily attacked by enzymes. Collagen fibrils in areas of inflammation, like those altered by ethylene oxide, might accordingly be considerably more susceptible to enzymatic digestion, *e.g.*, by local tissue cathepsins(10). This could mean also that the breakdown and

TABLE I. Digestion of Ethylene-Oxide-Treated Reconstituted Collagen by Single and Combined Pure Cultures of Oral Bacteria.

Cultures tested (strains)	% loss in total hydroxyproline
None	0
Diphtheroid bacilli (PC7, PC8)	20, 0
Fusiform " (F1, F3, F4, F5, F7)	0, 0, 16, 18, 0
Streptococci (PC1, PC2, PC6)	0, 0, 0
Veillonellae (V1, V2, V3, V4, V5)	0, 18, 18, 18, 0
Above cultures combined	77, 77
C. histolyticum	59

loss of collagenous fibers, as seen for example in periodontitis, results from primary alteration by the inflammatory response and secondary attack by bacterial proteases.

Summary. Treatment with ethylene oxide rendered reconstituted acid-soluble collagen readily digestible by combined pure cultures of human oral strains of fusiform bacilli, diphtheroid bacilli, streptococci, and veillonellae. The fact that only a few of these strains individually attacked such altered collagen to a slight extent indicates digestion by synergistic and symbiotic reactions between members of the oral microbiota. Concomitantly with increased digestibility from treatment with ethylene oxide, reconstituted collagen exhibited marked morphological changes as revealed by electron microscopy.

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Separation of 2 Glutamic-Oxalacetic Transaminases by Paper Electrophoresis.* (25469)

GERARD A. FLEISHER, CLARENCE S. POTTER AND KHALIL G. WAKIM
(With technical assistance of Marcella Pankow and David Osborne)
Mayo Clinic and Mayo Fn.,† Rochester, Minn.

The enzyme glutamic-oxalacetic transaminase (GOT) has attracted considerable interest recently because its concentrations in blood and tissue are altered characteristically in a number of disease states. Methods of purification devised up to now(1,2) have produced no indication that this enzyme may occur in more than one form. We observed that crude GOT in an extract of cardiac muscle from dog, when chromatographed on certain ion exchangers, behaves as a mixture of 2 enzymes. When CM-cellulose was employed with phosphate buffer of pH 7.0 and $\Gamma/2 = 0.01$ or less, one component was adsorbed and could be eluted at higher ionic strength and lower pH, while the other was adsorbed very little if at all. With DEAE-cellulose the behavior of the 2 fractions was reversed. These findings suggested that the 2 GOT's may have opposite electrical charges at pH 7. To test this assumption, the paper electrophoresis of crude preparations of GOT was investigated.

Methods and materials. Tissues were minced and extracted in a blendor with 3 volumes of cold water. The centrifuged extracts were concentrated several fold by ultrafiltration at 0°C. The solutions were recentrifuged at 30,000 g. They were then applied, in proportions of 10 to 40 μl, containing about 1 to 5 mg of protein, to the paper strips in a Spinco electrophoretic apparatus Model R. Phosphate buffer of pH 7.4, $\Gamma/2 = 0.075$, was used, often with the addition of one per cent bovine serum albumin as a stabilizer of enzymatic activity. Stabilization could be achieved also by applying quantities of tissue extracts corresponding to the larger amount of protein. Current was 5 milliamperes, temperature 8°C,

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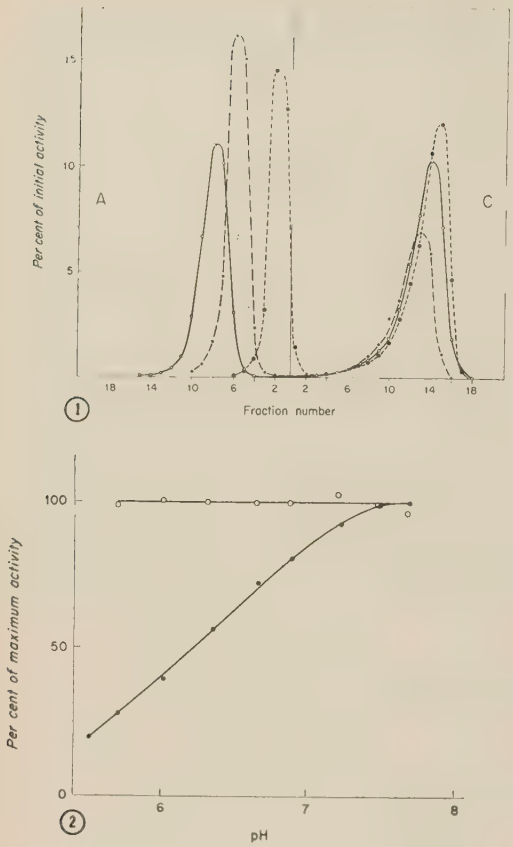

FIG. 1. Distribution of GOT activity following paper electrophoresis of crude heart extracts of man (○——○), pig (●– – –●), and dog (●·-·-●). Preparations were applied at center line. A and C represent, respectively, anode and cathode.

FIG. 2. Dependence on pH of the GOT activity (●, anionic; ○, cationic) of dog heart fractions.

time 45 hours. After electrophoresis the strips sometimes were dried partially in air (up to 1 hour), which did not cause loss of activity. They were cut into sections 5 mm wide, each of which was eluted with 2 ml of 0.9% NaCl. The various fractions were tested for GOT activity by the spectrophotometric method of Karmen at 38°C(3). Enzymatic activities were expressed as percentages of total activity applied to the paper. With the precautions specified, recoveries ranged from 70 to 80%. For study of the dependence of GOT activity on pH and on concentrations of α-ketoglutarate and L-aspartate, use was made of electrophoretic fractions at the peak of the bands. The pH and substrate concentrations listed were those of the final test mixtures. For the differential test the conditions were pH 6.0 with 5 x 10^{-3} moles/l of aspartate and pH 7.4 with 10^{-1} moles/l of aspartate. In both tests the α-ketoglutarate was 2 x 10^{-2} moles/l, and the buffer concentration and all other conditions were those of the standard test at pH 7.4. Because of the difference in substrate concentrations, the results in the special test at pH 7.4 were about 16% higher than in the standard test.

Results and comment. Electrophoretic patterns of GOT in heart extracts of man, pig, and dog are shown in Fig. 1. In each instance there were 2 separate active fractions of which one migrated toward the anode, the other toward the cathode. In the anionic activity the human transaminase always moved the fastest, the canine transaminase the slowest. On the other hand, differences in the rates of migration among the cationic enzymes are not necessarily significant. The ratios of the sum of cationic to the sum of anionic activities were 1.15, 0.72 and 1.37, respectively, for human, porcine, and canine heart. These patterns were obtained with 1 to 2 mg of tissue protein in the presence of serum albumin. In the absence of albumin the cationic bands were reduced to less than half, while the anionic bands were not affected. Increasing the amount of tissue protein applied to the paper was found to have the same beneficial effect as the presence of albumin in the buffer.

The 2 bands of GOT activity behave differently toward changes in pH shown in Fig. 2 for dog heart. The activity of anionic GOT, relatively low at pH 6 and less, rises to a maximum near pH 7.7, while the cationic GOT has a constant activity over this pH range. The former curve corresponds to that obtained with human serum(3) and with purified enzyme from pig heart(1).

The dependence of the 2 enzymatic activities on substrate concentrations was investigated at pH 6.0 and 7.4. The Michaelis constants determined from Lineweaver-Burk plots are summarized in Table I. The constants for L-aspartate at optimal concentration of α-ketoglutarate show striking differences, indicating that cationic enzyme has much greater affinity than the anionic for this substrate. On the other hand the latter enzyme

TABLE I. Michaelis Constants for 2 GOT Fractions from Canine Heart.

GOT fraction	pH	Km for α-ketoglutarate, moles/l*	Km for L-aspartate, moles/l†
Cationic	6.0	1.08×10^{-3}	2.48×10^{-3}
	7.4	$1.18 \times$ "	$.70 \times$ "
Anionic	6.0	$.17 \times$ " ‡	$38.4 \times$ " §
	7.4	$.57 \times$ "	$11.9 \times$ "

* With 66.7×10^{-3} moles/l of L-aspartate.
† " $13.3 \times$ " " " α-ketoglutarate.
‡ Inhibition above 4×10^{-3} moles/l of α-ketoglutarate.
§ Km = 21.9×10^{-3} moles/l at 3.33×10^{-3} moles/l of α-ketoglutarate.

showed greater affinity for α-ketoglutarate than the former, but was inhibited at pH 6.0 by concentrations above 4 x 10^{-3} moles/1.

On the basis of these data a differential test at these 2 pH values was devised for the purpose of calculating the proportion of the 2 GOT activities in unfractionated extracts. At pH 7.4 the sum of both activities was measured with an excess of both aspartate and α-ketoglutarate; at pH 6.0 cationic GOT was determined in almost complete exclusion of anionic GOT by using a high concentration of α-ketoglutarate and a low concentration of aspartate. The activity quotients (activity at pH 6.0 divided by activity at pH 7.4) are summarized in Table II. They were 0.415 to 0.472 for cationic fractions of cardiac muscle and liver from different species, and 0.023 to 0.027 for anionic fractions. The quotients found in crude extracts of heart (Table III) indicate a distribution comparable to that which the electrophoretic separation of similar extracts had shown (Fig. 1). It is of interest

TABLE II. Activity Quotients (Results at pH 6.0/Results at pH 7.4) of Cationic and Anionic GOT.

Source		Quotient: Cationic GOT	Quotient: Anionic GOT
Heart	Dog	.452	.026
	Man	.465	.026
	Pig	.415	.023
Liver	Dog	.472	.027

that the ratio between activities in canine and human liver is similar to the ratio between activities in the 2 kinds of heart, while serum from 3 patients with myocardial infarction and one with acute viral hepatitis gave indications for only small portions of cationic transaminase (Table III).

We have also studied the electrophoretic behavior of glutamic-pyruvic transaminase in heart and liver. There was no indication, however, for the presence of more than one component.

TABLE III. Activity Quotients (Results at pH 6.0/Results at pH 7.4) of Extracts and Serums, and Calculated Portions of Cationic Transaminase.

Source		Quotient	Cationic GOT, %
Crude extracts			
Heart	Dog	.281	60
	Man	.241	49
	Pig	.203	46
Liver	Dog	.255	51
	Man	.248	51
Serums			
Myocardial infarction		.057	7
		.067	9
		.074	11
Infectious hepatitis		.053	6

We are presently engaged in purification of cationic GOT.

Summary. By use of paper electrophoresis of crude extracts of heart and liver, 2 fractions with GOT activity were obtained, one of which migrated toward the anode and showed the known characteristics of serum GOT, while the other migrated toward the cathode and exhibited different affinities for both α-ketoglutarate and L-aspartate.

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Transplantation of Leukemoid Leukocytes in Irradiated Mice. (25470)

L. H. Smith, R. A. Popp and W. St. Amand* (Introduced by Charles C. Congdon)
Biology Division, Oak Ridge National Laboratory,† Oak Ridge, Tenn.

Transplantation and proliferation of infused bone marrow in several different species of lethally X-irradiated animals is well established(1); stem cells are believed to be involved. It is not known, however, whether one or more types of stem cells participate. One approach lies in the use of preparations containing fewer cell types than bone marrow. Isolation of a near-homogeneous cell population from bone marrow suspension by differential centrifugation has been successful, but not to the extent of concentrating or clearly identifying an effective cell type(2). That leukemoid leukocytes of leukemoid blood‡ improve 30-day survival of lethally irradiated recipients suggested the potential usefulness of these cells better to define the cell type(s) responsible for transplantation because (a) there appear to be fewer cell types in leukemoid blood than in bone marrow, and (b) there is evidence that these cells proliferate in irradiated mice in a manner similar to that of bone marrow(3,4). About 90 to 95% of leukemoid leukocytes are mature, normal-looking granulocytes that presumably are not capable of further division and therefore would not be able to transplant and proliferate. The remaining leukocytes are small lymphocytes and various sized mononuclear cells with a spherical or irregular nucleus. Leukemoid blood does not appear to contain nucleated erythrocytic cells although the reticulocyte count is frequently elevated. Megakaryocytes have never been observed in leukemoid blood. The purpose of our study was to clarify further the potential of leukemoid leukocytes to transplant and give rise to erythrocytes in lethally irradiated mice. A preliminary autoradiographic study was made of these leukocytes that had been incubated *in vitro* with H^3-thymidine.

Methods. Both SeC§ and BALB/c strains of mice were exposed to a dose of 250-kv X rays‖ that was 100% lethal to control animals by day 14 (*i.e.*, SeC 800 r, BALB/c 750 r). Leukemoid leukocytes were separated from tumor-bearing mice [SeC or (BALB/c × A) F_1] by sedimentation of erythrocytes with dextran.¶ Within 4 hours after irradiation, each experimental mouse (BALB/c or SeC) received intravenously ~ 100×10^6 nucleated homologous leukemoid leukocytes. Popp *et al.* (5) have shown that genetic differences in hemoglobin patterns can be used as markers to identify donor- and host-type erythrocytes in irradiated mice given homologous bone marrow. Therefore, hemoglobin from tail blood samples was subjected to starch gel electrophoresis at several intervals after treatment. The hemoglobin patterns were characterized as single type, diffuse type, or mixtures of single and diffuse. The SeC strain has a single type hemoglobin pattern and the BALB/c, or the hybrid (BALB/c × A)F_1, has a diffuse type. *In vitro* uptake of H^3-thymidine by leukemoid leukocytes was studied autoradiographically. Freshly drawn leukemoid blood was incubated (~ 24°C) with H^3-thymidine (1.0 μc/ml of whole blood). Blood smears were made at hourly intervals up to 7 hours. Smears were dipped in NTB 2 liquid emulsion, exposed for 14 days, and stained with Wright-Giemsa.

Results. In 3 lethally irradiated SeC mice given leukemoid leukocytes from (BALB/c × A)F_1 donors, the percentage of diffuse-type hemoglobin was 0% on day 15, ~ 25% on day 46, and 100% by day 77. The hemo-

* Present address: Dept. of Biology, Univ. of Mississippi, University, Miss.

† Operated by Union Carbide Corp. for U. S. Atomic Energy Comm.

‡ Growth of a particular transplantable squamous cell carcinoma in the mouse produces an extreme granulocytosis (leukemoid reaction)(6). These leukocytes are referred to as leukemoid leukocytes.

§ Obtained from Dr. W. L. Russell of the Biology Division, Oak Ridge National Lab., Oak Ridge, Tenn.

‖ hvl, 0.5, mm of Cu; ~ 160 r/min; 15 ma.

¶ Grade HH; average mol. wt. 275,000; 3% in Tyrode's solution; R. K. Laros Co., Bethlehem, Pa.

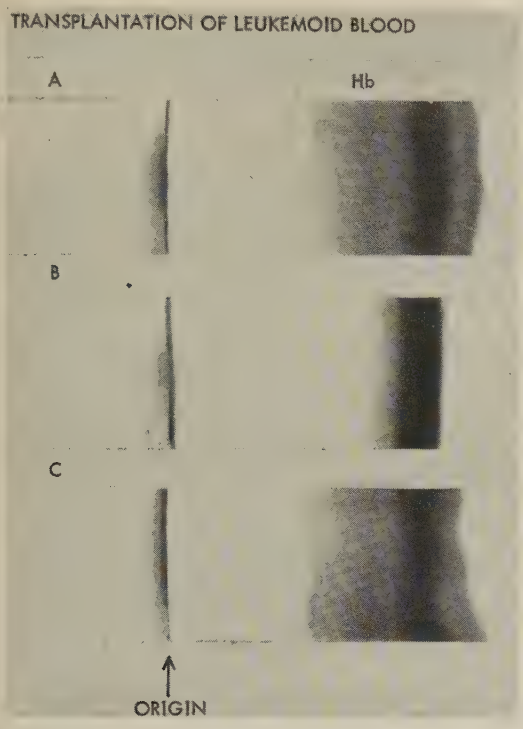

FIG. 1. Starch gel electrophoresis patterns of hemoglobin: A, normal (BALB/c × A)F_1; B, normal SeC; C, 800 r SeC given leukemoid leukocytes from (BALB/c × A)F_1, 77 days after treatment.

globin pattern for one of these SeC mice (77 days after treatment) and the control patterns are shown in Fig. 1. A similar appearance rate of donor-type hemoglobin was observed in 2 irradiated BALB/c mice given leukemoid leukocytes from SeC tumor-bearing mice.

After 1 and 7 hours incubation with H^3-thymidine, about 1 and 2.5%, respectively, of leukocytes of leukemoid blood were labeled. Several cell types incorporated the isotope, principally mononuclear cells and granulocytes with a ring nucleus (Fig. 2). Many of the labeled mononuclear cells stained poorly and could not be further characterized. Most labeled cells contained 10 to 60 grains. Small lymphocytes and segmented polymorphonuclear cells were not labeled. There was no apparent change in the spectrum of cell types labeled as incubation time was increased.

Discussion. The data show that, in the circulation of mice with a leukemoid condition, there are cells capable of transplanting and giving rise, through division and differentiation, to mature erythrocytes in lethally irradiated recipients. From indirect evidence, it was suggested that transplantation of leukemoid leukocytes in irradiated recipients occurs in a manner similar to that of bone marrow(3). Merwin(4), using a Harderian gland rejection method, obtained direct proof that hematopoietic tissue is repopulated by leukemoid blood. She interpreted her results to mean that many of the marrow and lymph node cells of donor type proliferated in irradiated mice. We found (Smith and Congdon, unpublished observation) that recovery of peripheral blood cell elements is the same whether isologous bone marrow or leukemoid leukocytes are given. It is conceivable, therefore, that certain leukemoid leukocytes are potential precursors for all types of leukocytes and for platelets as well as erythrocytes.

According to results of many recent studies, *in vitro* uptake of H^3-thymidine by certain types of leukemoid leukocytes indicates that DNA synthesis occurred in these cells. A few of these labeled elements are large mononuclear cells that have staining characteristics of young forms—*viz.*, nucleus containing finely divided chromatin, deeply basophilic cytoplasm. Incorporation of thymidine by some of these cells suggests that they are responsible for the transplantation phenomenon in lethally irradiated recipients. Our data show, in addition, that some granulocytic cells with

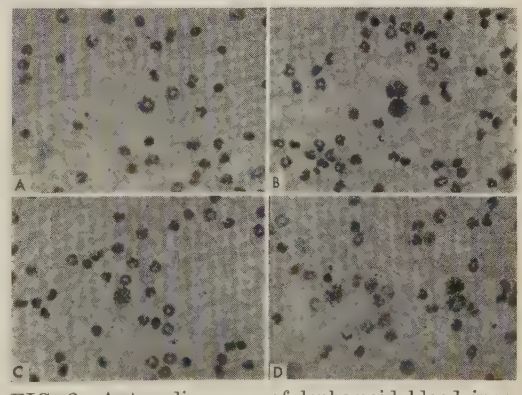

FIG. 2. Autoradiograms of leukemoid blood incubated 1 hr with H^3-thymidine *in vitro*; 14-day exposure. A, ring granulocyte; B, mononuclear cells; C, mononuclear cell with immature characteristics; D, ring granulocyte and 2 mononuclear cells.

a ring-form nucleus also incorporate thymidine. Whether these cells are young or mature cannot be determined decisively. Some of these cells have a pale cytoplasm and a nucleus containing coarsely divided chromatin (mature forms); others have a basophilic cytoplasm and a nucleus containing finely divided chromatin (young forms). If we assume that mature forms are not able to divide, thymidine incorporation into these cells would indicate ploidy, or possibly turnover of thymidine, rather than potential to divide. It is not likely that the labeled ring forms arose from labeled precursors since the number of labeled rings did not increase as incubation time increased, nor was there a reduction in the number of grains over the ring forms.

Summary. Using hemoglobin markers to identify donor- and host-type erythrocytes, we have demonstrated that cells in the leukocyte fraction of leukemoid blood are capable of transplanting and giving rise to mature erythrocytes of donor type in lethally irradiated mice. Autoradiograms showed that several cell types in leukemoid blood incorporate H^3-thymidine *in vitro*.

We wish to thank Mr. W. D. Gude of the Biology Division for making the autoradiographs.

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Augmentation of Chlortetracycline Activity Against Two Strains of *Staphylococcus* by Kinetin.* (25471)

ROSS H. HALL† AND GEORGE O. GALE (Introduced by T. H. Jukes)
Nutrition and Physiology Dept., Lederle Labs., American Cyanamid Co., Pearl River, N. Y.

Kinetin (6-furfurylaminopurine) was shown by Miller *et al.*(1,2) to be a cell division factor for tobacco pith tissue in tissue culture. This compound, originally isolated from aged or heated deoxyribonucleic acid (DNA) by these workers, was shown by Hall and de Ropp(3) to be essentially an artefact with respect to DNA. The latter workers established that the presence of kinetin in DNA was due to fortuitous interaction of adenine with 2-deoxyribose. Since the discovery of kinetin, a number of reports have described the influence of this compound and related compounds in several diverse biological systems. In the bacterial field, Lansford *et al.* (4) have shown that kinetin and certain other 6-(substituted amino) purines will augment inhibition of *Lactobacillus arabinosus* by 2,4-diamino-6,7-diphenylpteridine. Braun(5) has demonstrated that kinetin will shift the population of a culture of mixed rough and smooth *Brucella suis* in favor of the smooth cells. This shift is the reverse of that in the absence of kinetin. The increasing incidence of infections caused by antibiotic-resistant staphylococci has prompted us to examine the effects of kinetin and related compounds on these microorganisms. In this paper we wish to report that kinetin (and some analogs) will enhance the action of chlortetracycline (CTC) against an antibiotic-resistant strain of staphylococcus *in vitro*.

Materials. Kinetin, 6-(3-pyridylmethyl-

* We are indebted to G. S. Redin and M. E. McCoy for permission to include their *in vivo* data. We acknowledge suggestions and encouragement of Drs. J. A. Brockman and T. H. Jukes throughout this work.

† Present address: Dept. of Exp. Therapeutics, Roswell Park Memorial Inst., Buffalo, N. Y.

TABLE I. Effect of Kinetin and Its Derivatives on Bacteriostatic Action of Chlortetracycline for *S. albus* 69R.

Compound	Conc., γ/ml, of compound	No. of colonies ($\times 10^{-8}$)/ml			
		Saline control	Compound *per se*	CTC *per se*, 50 γ/ml	Compound + 50 γ/ml of CTC
Kinetin	.1	2.47	2.12	2.09	.00
	1.0	2.47	2.42	2.09	.00
	4.0	2.35	2.22	2.09	.70
Kinetin riboside	4.0	2.43	2.40	2.50	.85
6-(3-pyridylmethylamino)-purine	4.0	2.43	2.43	2.50	1.69
6-(3-pyridylmethylamino)-9-D-ribofuranosylpurine	4.0	2.43	2.44	2.20	1.69
6-(Dimethylamino)-9-D-(3-deoxy-3-amino)-ribofuranosylpurine	4.0	2.43	2.40	2.51	.50
6-(Dimethylamino)-9-D-ribofuranosylpurine	5.0	2.26	2.46	2.14	1.35
Adenosine	5.0	2.35	2.37	2.66	2.37

amino) purine, 6-(2-pyridylmethylamino) purine, 6-benzylaminopurine, 6-(N-methylbenzylamino) purine, and 6-furfurylthiopurine were obtained from Dr. M. W. Bullock (6). Kinetin riboside(7), 6-(3-pyridylmethylamino)-9-D-ribofuranosyl purine(7), 6-dimethylamino-9-D-ribofuranosyl purine(8) and 6-dimethylamino-9-(D-3-deoxy-3-amino) ribofuranosyl purine(9) were obtained through Dr. H. Kissman. The 2 indole analogs, 6-[(β-indoyl-3-ethyl)-amino] purine, 6-{[β-(5-methyl-3-indoyl) ethyl] amino} purine, were synthesized in this laboratory by Mr. R. Haselkorn using a general method(6) whereby the corresponding indoylethylamine‡ was condensed with 6-chloropurine. The microorganism used throughout the course of the experiments was *Staphylococcus albus* 69R (*Micrococcus pyogenes* var. *albus* 69R). It was a hospital isolate obtained from Mr. A. C. Dornbush of these laboratories.

Methods. Tubes of trypticase-soy (TS) broth containing the adducts (total vol. 6 ml) were warmed to 37° then inoculated with 0.1 ml of a 1 x 10^{-2} dilution of a 6 hour (late exponential phase) TS broth culture of *S. albus* 69R. After 24 hours incubation at 37° the cultures were serially diluted and plated on to TS agar. The plates were incubated for 24 hours at 37°. Four plates were made per tube and tubes were run in duplicate or triplicate. Because of the tendency of staphylococci to clump, the plate counts reported in Table I are essentially viable units/ml. The growth curves were run with TS broth cultures started as above except 18 hour cultures were used for the inoculum. Optical density readings were made at 610 mμ. *In vivo infection.* Infections were produced in CFI white mice, females 17-20 g (Carworth Farms) by intraperitoneal injection of 0.5 ml of a 1 x 10^{-2} dilution of 5 hour TS blood broth cultures of *Micrococcus pyogenes* var. *aureus*, strain Smith. Kinetin and the antibiotics were suspended in 0.2% aqueous agar. Eleven groups of 10 mice each were pretreated with kinetin by subcutaneous injection with 0.5 ml doses of 64 mg/kg/dose at 24, 6 and 0.5 hours prior to infection. Within 0.5 hour after injection the mice were treated subcutaneously with CTC or tetracycline in graded doses. A parallel series of non-kinetin treated animals was run simultaneously plus the following controls. One group treated with kinetin but not infected, 2 groups not treated with kinetin but infected, and one group not treated with kinetin and not infected. Heart blood cultures were made routinely from mice dying 6 days postinfection. All such mice showed the infecting organism.

Results. Staphylococcus albus 69R will grow in broth culture in presence of at least 125 γ/ml of CTC. The data in Table I show growth of this microorganism as reflected by plate counts in the presence of CTC or kinetin, and in the presence of a combination of

‡ We are indebted to Dr. S. Davis for supplying indole precursors used in these syntheses.

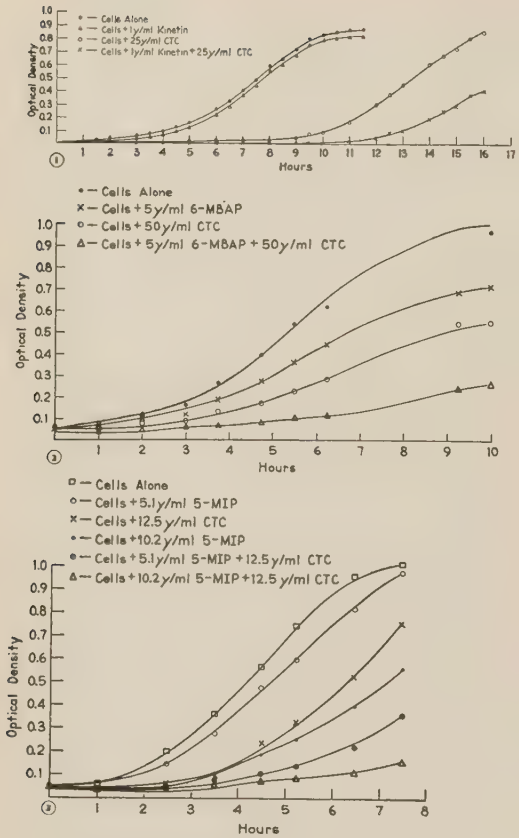


FIG. 1. Effect of kinetin on the response of *S. albus* 69R to CTC.

FIG. 2. Effect of 6-(methylbenzyl)aminopurine (6-MBAP) on the growth of *S. albus* 69R in the presence and absence of CTC.

FIG. 3. Effect of 6-{[2-(5-methyl-3-indolyl)ethyl] amino}-purine [5-MIP] on the growth of *S. albus* 69R in the presence and absence of CTC.

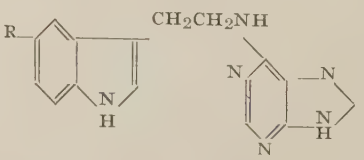
the two. Neither kinetin nor CTC by themselves influenced plate counts after 20 hours of growth, but the combination reduced cell counts by at least 100-fold. Other kinetin analogs listed in Table I gave the same result to a lesser degree. There was no advantage in using kinetin riboside and neither did the riboside of 6-(3-pyridylmethylamino)-purine have an advantage over the free base. Adenosine was inactive at the level tested.

A more accurate picture of the potentiation of CTC by kinetin was obtained by studying turbidimetric growth curves of *S. albus* 69R in presence of CTC and the kinetin compounds. Fig. 1 shows that 25 γ/ml of CTC alone extends the lag phase of growth, although exponential growth rate and total growth were essentially the same as the control. This effect *per se* of sublethal amounts of antibiotics on growth of resistance cells has been studied in detail(10). For our purposes here the relative lag periods are of interest. Kinetin by itself at a level of 1 γ/ml had no effect on the course of growth of *S. albus* 69R, but in combination with CTC the length of the lag phase was considerably extended over that of CTC alone. These data show that plate count differences reported in Table I are not necessarily differences between total growth but differences between control cultures at maximum growth and experimental cultures still in late exponential phase.

This phenomenon was also studied with the kinetin analog, 6-(N-methylbenzylamino) purine (6-MBAP) (Fig. 2). This compound itself at a level of 5 γ/ml was slightly toxic to *S. albus* 69R as evidenced by a slight increase in the lag phase of growth. In combination with CTC it was synergistic. Thus apart from the inherent toxicity of 6-MBAP there seemed to be a potentiation of CTC activity analogous to that of kinetin.

Similar studies were made with 6-benzylaminopurine, 6-furfurylthiopurine, 6-(2-pyridylmethylamino) purine and 6-methylaminopurine. In each case no toxicity was noted at 1-10 γ/ml level and there was no potentiation of CTC action.

A different class of kinetin analogs was studied next, namely, indoylethylaminopurines. The parent compound, 6-[(β-indoyl-3-ethyl)-amino] purine I was first prepared by Lettré(11) who reported that it was toxic to fibroblasts in tissue culture. This particular compound at a level of 10 γ/ml in the cul-



ture had no influence on the shape of the *S. albus* 69R growth curve nor did it potentiate CTC. But the 5-methyl analog 6-{[β-(5-methyl-3-indoyl)ethyl] amino} purine II was slightly inhibitory to *S. albus* 69R

TABLE II. Effect of Pre-infection Treatment with Kinetin upon Activity of Subcutaneously Administered Chlortetracycline and Tetracycline against Staphylococcus Smith Infection in Mice. (Pooled results of 4 separate tests.)

Antibiotic dose, mg/kg	Survival on 14th day post-infection: Without kinetin pretreatment Alive/Total	% effect	With kinetin pretreatment Alive/Total	% effect
	Chlortetracycline treated mice			
2.0	38/40	95.0	40/40	100.0
1.0	29/40	72.5	37/40	92.5
.5	16/40	40.0	26/40	65.0
.25	4/40	10.0	8/40	20.0
.125	2/20	10.0	1/20	5.0
	Tetracycline treated mice			
4.0	37/40	92.5	40/40	100.0
2.0	26/40	65.0	35/40	87.5
1.0	20/40	50.0	27/40	67.5
.5	5/40	12.5	13/40	32.5
.25	0/20	.0	1/20	5.0

Kinetin controls: 95% (38/40) kinetin pretreated-infection control mice were dead within 2 days post-infection.

Infected controls: 100% (80/80) untreated-infected control mice were dead within 2 days post-infection.

Age-conditioned controls: 97.5% (39/40) untreated-uninfected control mice were *alive* on the 14th day when test terminated.

at levels of 5-10 γ/ml (Fig. 3). A combination of this compound with CTC was synergistic as shown by the increased lag phase of growth.

Kinetin and most of the analogs tested were relatively non-toxic to *S. albus* 69R, yet in some way they potentiated the inhibitory action of CTC. We have no evidence as to why this occurs; however, the effect would seem to be more than an anti-metabolic one. The question arises whether the effect is related to stimulation of cell division by kinetin in some plant systems(1,2,3). Guttman(12,13), in a cytological investigation of meristematic onion root cells grown in solutions of kinetin found that kinetin seemed to influence metabolism of nuclear ribonucleic acid. Perhaps there is an analogous effect in staphylococci that makes the cell more vulnerable to the action of CTC. The potentiation of CTC by kinetin was not only observed *in vitro* but was also demonstrated in an *in vivo* system. In cooperation with Mr. G. Redin and Miss E. McCoy, studies were made with an infection of *Micrococcus pyogenes* var. *aureus*, strain Smith, in mice. This is a CTC-sensitive organism. Its use was necessitated by the unavailability of a mouse-virulent CTC-resistant staphylococcus. Data in Table II show that the activity of both CTC and tetracycline was augmented by treating the mice with kinetin prior to infection. Application of the statistical method of Litchfield and Wilcoxon (14) for evaluation of dose-effect experiments showed that the combination of CTC and kinetin was 1.5 times as active as CTC alone (95% confidence limits 1.1-2.0) and the tetracycline-kinetin combination was 1.8 times as active as tetracycline alone (95% confidence limits 1.3-2.5). These data demonstrate first, that kinetin will enhance the activity of the tetracyclines *in vivo* and second, that enhancement of antibiotic activity by kinetin also occurs with an antibiotic-sensitive staphylococcus.

Summary. 1. Kinetin, although non-toxic to an antibiotic-resistant strain of *Staphylococcus albus*, augmented the action of chlortetracycline against this microorganism *in vitro*. 2. Some other 6-(substituted amino) purines had this property. 3. Kinetin caused a slight but statistically significant increase in activity of chlortetracycline and tetracycline against an antibiotic-sensitive strain of *Staphylococcus aureus* in mice.

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Serum Lipoproteins in Atherosclerosis-Susceptible and Resistant Pigeons.*† (25472)

HUGH B. LOFLAND AND THOMAS B. CLARKSON (Introduced by Camillo Artom)
Dept. of Biochemistry, and Vivarium, Bowman Gray School of Medicine, Wake Forest College, Winston-Salem, N. C.

White Carneau pigeons (both male and female) develop spontaneous atherosclerosis involving aorta and coronary arteries, while Show Racer pigeons appear resistant to atherosclerosis(1,2). Despite striking differences in incidence of the disease, the 2 breeds do not differ significantly in serum levels of total lipides, cholesterol (free or ester), or phospholipides(3). Studies on man(4,5,6) and experimental animals(7,8,9) indicated that atherosclerosis may be associated with changes in levels of serum lipoproteins, especially increases in beta-lipoproteins. Accordingly, we thought it of interest to compare atherosclerosis-susceptible and resistant pigeons for possible differences in levels of beta-lipoproteins, as determined by agar precipitation method of Boyle and Moore(10). It soon became apparent that enormous fluctuations occurred in the K-agar Δ‡ values obtained for female pigeons. These results, and those of a more detailed study of serum lipides in pigeons are reported here, together with observations on reliability of the K-agar precipitation technic.

Materials and methods. White Carneau and Show Racer pigeons ranging in age from 6 weeks to 7 years from Palmetto Pigeon Plant, Sumter, S.C., were maintained on commercial pigeon rations (mixtures of corn, peas, wheat, kaffir, and minerals). Blood samples were obtained from the alar vein. On one sample of serum (within one to 5 hours after drawing), the K-agar Δ values were determined(10). Another sample of whole blood was mixed with ACD§ solution and centrifuged, and the supernatant subjected to low-temperature alcohol fractionation, as described by Lever, *et al.*(11). This procedure yielded 2 fractions, one containing alpha-lipoproteins, and the other beta-lipoproteins. Lipides were extracted from each fraction with boiling alcohol and alcohol-ether, and purified with chloroform(12). "Total lipides" of each extract were determined by weight and an aliquot was used for determination of total cholesterol by the method of Zlatkis, *et al.* (13), as modified by Rosenthal, *et al.*(14). On another aliquot, lipide phosphorus was determined according to Stewart and Hendry (15).

Results. Values for K-agar Δ and total cholesterol in serum of sexually mature pigeons of the 2 breeds are shown in Table I. These birds were actively reproducing. No significant difference in levels of beta-lipoprotein exists among males of the 2 breeds. The extremely wide range of K-agar Δ values determined on female birds, however, suggested that these values were influenced by sequence of events in the ovulatory cycle, thus making difficult any comparison we could make between females of the 2 breeds. This suggestion is supported by results of K-agar Δ de-

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† The authors are indebted to Dr. J. S. King for electrophoretic analyses, and to Katherine King for valuable technical assistance.

‡ K-agar Δ is defined as optical density of sample of serum mixed with buffer and a solution of K-agar, minus optical density of a blank consisting of serum plus buffer.

§ ACD Solution: Citric acid monohydrate 0.8%, sodium citrate ($2H_2O$) 2.2%, and dextrose 2.2%.

TABLE I. Serum Beta-Lipoproteins and Total Cholesterol in 2 Breeds of Pigeons.

Breed	No. of birds	Avg age	Sex	Beta-lipoproteins* Range	Beta-lipoproteins* Avg and S.E.	Total cholesterol, mg %†
White Carneau	18	2.8 yr	♂	20- 69	34.9 ± 2.8	407.2 ± 14.9
	11	1.4 "	♀	24-148	78.4 ± 16.4	468.5 ± 23.0
Show Racers	11	1.7 "	♂	13- 61	27.8 ± 4.1	390.3 ± 24.9
	18	3.3 "	♀	19-512	173.7 ± 44.8	434.7 ± 25.7
White Carneau	130	6 wk	Mixed	11- 90	40.4 ± 3.8	401.4 ± 15.9

* K-agar Δ value (see footnote 1). † Avg value and stand. error of mean.

terminations on 130 six-week-old White Carneau pigeons of both sexes. From these results, also included in Table I, it appears that sexually immature birds have serum lipoprotein values very similar to those of older males.

To gain more information on changes in serum lipides during the ovulatory cycle, these changes were followed systematically in 2 pairs of mated White Carneau pigeons. Beta-lipoprotein cholesterol (expressed as % total cholesterol) and K-agar Δ values for one pair of birds are shown in Fig. 1. In the male, only about 30% of total cholesterol is carried in the beta fraction, and low K-agar Δ values are found consistently. The female, on the other hand, shows a sharp rise in both beta-lipoprotein cholesterol and K-agar Δ values approximately 30 days following egg laying. It appears that there is a shift in transport of cholesterol from alpha to beta fraction at this time. Shortly after these changes occurred, a new egg was laid.

Further evidence for the striking difference between male and female in quantitative aspects of lipide transport was obtained by subjecting serum samples, taken 30 days after egg laying, to electrophoresis in Spinco Model H Electrophoresis apparatus (Fig. 2). Differences between male and female patterns are obvious, and are similar to those described for chickens by Moore(16).

In addition to cholesterol, serum total lipides and phospholipides also undergo a shift from alpha to beta lipoprotein (Fig. 3, in which the values (obtained from a different female pigeon) are expressed as per cent of lipide component present in the beta-lipoprotein). In the female, however, there is no positive correlation between K-agar Δ values and total cholesterol in serum. Whereas serum total lipides and phospholipides rise sharply at time of egg laying, there is no accompanying rise in serum total cholesterol (Fig. 4). Boyle and Moore state that in humans K-agar values are positively correlated with total serum cholesterol. It might be pointed out, of course, that there is no assurance that the substances precipitating with K-agar in human serum are qualitatively and quantitatively identical with those obtained by the same technic from pigeon serum.

Discussion. It has long been known that in serum of birds, lipides are raised considerably during egg-laying activity(17,18,19,20). Our data suggest that female pigeons, during their reproductive life, undergo regular periodic shifts in lipide transport from alpha- to beta-lipoprotein. These shifts are accompanied by increases in total phospholipides and by marked decreases in cholesterol/phospholipide ratios. It seems logical to assume that the changes observed are under the influence of changes in estrogen production. Somewhat similar changes in distribution of lipides between various lipoprotein fractions in serum of hens during the ovulatory cycle are also apparent from data of McIndoe(19). This author describes appearance of a new lipoprotein fraction just prior to egg-laying. This fraction may possibly be the precursor of a dense lipoprotein isolated from egg yolk by Schjeide and Urist(20).

Pick, *et al.*,(21) reported that cholesterol-fat fed chicks exhibited high cholesterol/phospholipide ratios, and extensive coronary atherosclerosis. When these chicks were treated with estrogen, they were free of coronary lesions and the cholesterol/phospholipide ratio was lowered, suggesting a protective action of estrogen against coronary atherosclerosis. It appears that this view cannot

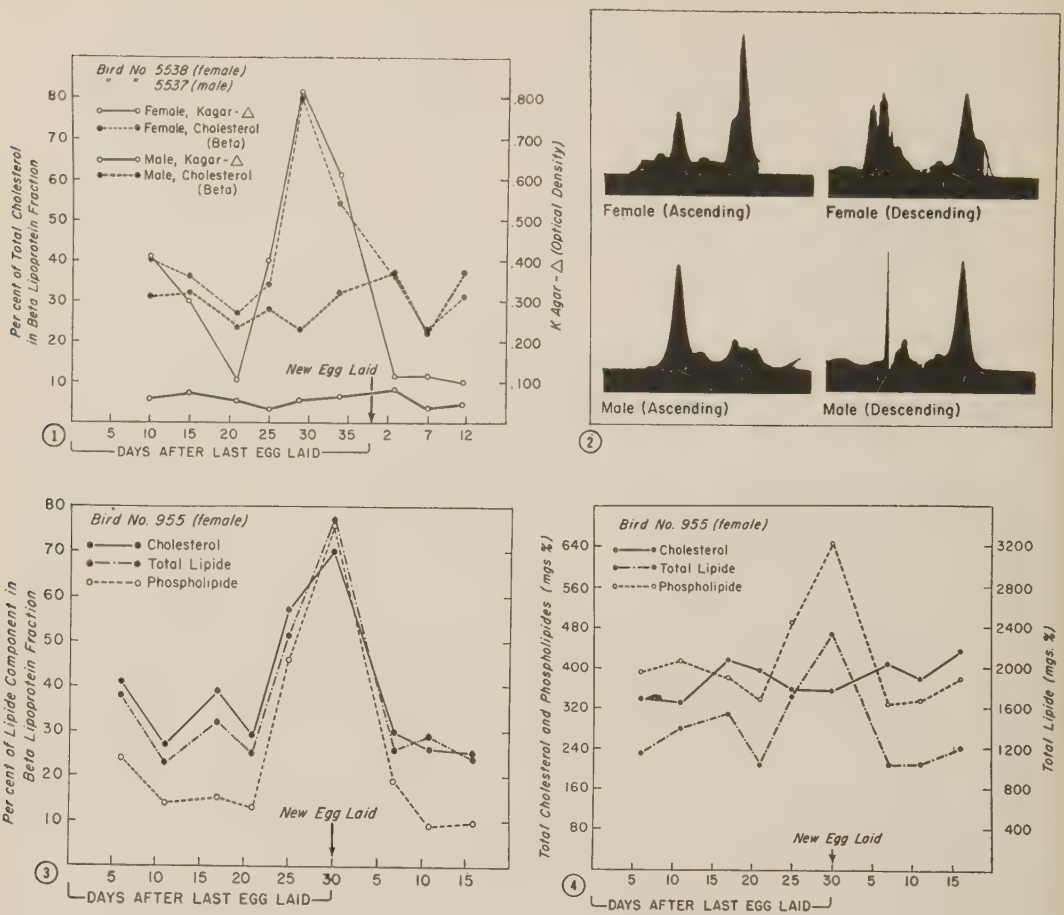

FIG. 1. Comparison of K-agar △ values, and beta-lipoprotein cholesterol in one pair of White Carneau pigeons during an ovulatory cycle.

FIG. 2. Electrophoresis of serum from male and female White Carneau pigeons. Blood samples drawn 30 days after last egg was laid.

FIG. 3. Changes in % of cholesterol, total phospholipides, and total lipides carried in beta-lipoproteins during ovulatory cycle of female White Carneau pigeon.

FIG. 4. Changes in total cholesterol, total phospholipides and total lipides in serum of a female White Carneau pigeon during an ovulatory cycle.

be easily extended to spontaneous atherosclerosis of pigeons. We previously reported lack of sex difference (hence estrogen protection) in incidence of aortic atherosclerosis in White Carneau and Autosexing King pigeons (3), and recently included coronary atherosclerosis in the same age group(2). Furthermore, from previous(3) and present data, it appears that although cholesterol/phospholipide ratio decreases periodically in female pigeons, these birds are not protected against atherosclerosis. Differences between results of Pick *et al.*,(21) and ours might be a reflection of the fundamental difference between the disease produced by cholesterol feeding and that occurring spontaneously in pigeons.

We compared these findings with the statement that in human beings, high levels of beta-lipoprotein cholesterol in serum may be associated with occurrence of myocardial infarction(5). In man, estrogen administration results in increased concentration of cholesterol in alpha-lipoproteins(22), while in the female pigeon the response to increased production of estrogen is in the opposite direction. Lack of a sex difference in incidence of atherosclerosis in pigeons makes doubtful any causal relationship between altered distribu-

tion of lipides in lipoproteins and atherogenesis in this species.

Summary. 1) Using the agar precipitation method and low temperature fractionation for determination of beta-lipoproteins, amounts of beta-lipoproteins, total lipides, phospholipides, and cholesterol in serum of atherosclerosis-susceptible and resistant breeds of pigeons were compared. The agar precipitation method seems a reliable method for estimating levels of beta-lipoprotein cholesterol in serum. 2) No significant differences were seen when male birds of the 2 breeds were compared. Lipoprotein levels of females varied within wide limits, probably due to phospholipemia associated with egg-laying activity. Such activity is accompanied by increased proportion of cholesterol in beta-lipoproteins. At time of egg-laying, total lipides and total phospholipides (but not total cholesterol) are increased in the serum. Thus, such cyclic changes appear to produce a more "favorable" (*i.e.*, lowered) cholesterol/phospholipide ratio. In contrast to cholesterol-fat fed chicks, female pigeons of susceptible breed are not protected against aortic or coronary atherosclerosis.

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Renal Tubular Excretion of Riboflavin in the Chicken.* (25473)

BARBARA R. RENNICK (Introduced by Jay Tepperman)
Dept. of Physiology, State University of N. Y., Syracuse, N. Y.

The renal tubule has been demonstrated to be capable of excreting a number of organic bases by an active transport system. Tetraethylammonium(1,2), n-methylnicotinamide (3), tolazoline (Priscoline)(4), choline and thiamine(5) represent a few of these compounds, most of which are quaternary ammonium compounds. Studies of competitive inhibition among this series indicate that the substances are transported by a common system. Excretion by the renal tubule of the chicken has been demonstrated for all of these substances. This study describes the renal tubular excretion of riboflavin (6,7-Dimethyl-9-1(d-l'-ribityl-) iso-alloxazine) in the

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chicken and demonstrates that it shares the transport system for one of the organic bases previously studied.

Methods. The renal tubular excretory rates of the various compounds in question were determined in unanesthetized hens (1.8 to 3 kg) according to method previously described by Sperber(6), Lindahl and Sperber (7) and by Rennick *et al.*(2,8). The compounds were infused at a constant rate into the renal portal circulation of one kidney *via* the ipsilateral saphenous vein, urine being collected separately from ureters of the 2 kidneys during successive 10-minute periods. The rate of renal tubular excretion of any given compound, by the kidney on the infused side, is expressed as the Apparent Tubular Excretion Fraction (ATEF), according to the formula $\frac{Exc_I - Exc_C}{INF} \times 100$, in which Exc_I represents rate of total urinary excretion of the compound on the infused side, Exc_C denotes rate on the contralateral side, and INF represents rate of infusion, all expressed in the same units. Thus, the ATEF expresses excess of the infused compound excreted by tubules of the infused kidney as a percentage of amount infused. It should be pointed out that the renal portal circulation is not obligatory since only a portion of the saphenous venous blood is diverted to the ipsilateral kidney and its tubules. The proportion of blood thus diverted and the proportion which bypasses the ipsilateral kidney to enter the renal vein and thus the systemic venous circulation are controlled by a muscular valve interposed between renal portal and renal veins. As index of the proportion of saphenous venous blood, and hence of injected compound, which does enter the portal circuit, excretion of p-aminohippuric acid (PAH), infused simultaneously, was measured in all experiments at levels well below the Tm. Tubular transport rates of various quaternary compounds are therefore also expressed as the ratio of their ATEF values to that of PAH. Details of procedure are given in references cited. *Analyses of urine and infusion solution* for PAH were made by the method of Smith *et al.*(9). Riboflavin was determined by measuring transmittance in the Beckman DU at 450 mμ. The experiment was done in controlled light and analyses on urine samples and infusion were done immediately and protected from light.

TABLE I. Renal Tubular Excretion of Riboflavin (RIB) and PAH in the Chicken.

	ATEF			
	Avg		RIB / PAH	
	PAH	RIB		No. obs.
	45	13	.29	7
	88	36	.41	7
	65	27	.42	10
	69	23	.33	3
	64	25	.39	4
	67	18	.27	3
	62	20	.32	3
	54	25	.46	3
	70	19	.27	3
	70	33	.47	3
	53	29	.54	3
	42	20	.48	4
Avg	62	24	.39	53

Results. Riboflavin was infused into the renal portal circulation in the hen in doses of from 0.06 mol to 0.24 mol/minute. There was an ATEF for riboflavin of 24 or an absolute excess from the infused kidney of 24% as an average of 53 observations indicating tubular excretion of riboflavin (Table I). PAH administered simultaneously gave an ATEF of 62 and the ATEF $\frac{\text{Riboflavin}}{\text{PAH}}$ was 0.39. There was no consistent relationship between dose infused and ATEF resulting, so it was concluded that levels were not near Tm. The insolubility of riboflavin limited the range of concentrations used.

In an attempt to determine the transport system utilized by riboflavin, competitive inhibition by members of organic acid transport system and organic base transport systems were used. Benemid in a dose of 1500 γ/min after 30 minutes inhibited both PAH and riboflavin to 40% of original transport rate. As PAH and riboflavin were usually infused simultaneously there was the possibility that they were mutually inhibitory. When either one was separately infused, addition of the other did not affect ATEF. It was apparent that in the doses used they did not inhibit the transport of each other.

Quantitative comparison of organic base

TABLE II. Renal Tubular Excretion of Riboflavin and PAH in the Chicken and Inhibition of Riboflavin Excretion by Priscoline. Avg for 3 experiments.

	ATEF			
	PAH	RIB	RIB/PAH	No. obs.
	Before Priscoline			
	70	33	.47	3
	53	29	.54	3
	42	20	.48	4
Avg	55	27	.49	10
	After Priscoline			
	90	20	.22	4
	57	14	.25	3
	55	6	.11	4
Avg	67	13	.19	11

transport mechanisms have shown that tolazoline is one of the most potent competitive inhibitors within the system (10). When tolazoline was added to the infusion of riboflavin and PAH, excretion of riboflavin fell and PAH was not affected or rose. This was considered evidence of participation of riboflavin in the organic base transport system selectively. Fig. 1 shows the results of such an experiment. Table II describes results of 3 experiments in which tolazoline selectively inhibited riboflavin transport. In the dose of tolazoline used, 1.9 μM/min the tubular excretion of riboflavin was reduced 50%.

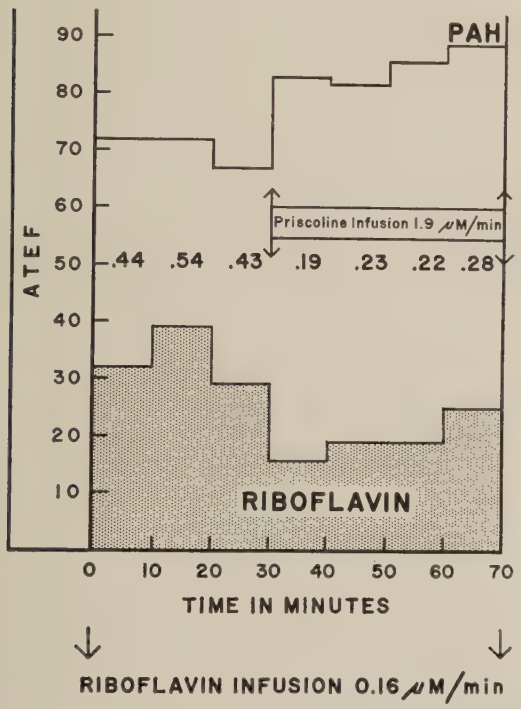

FIG. 1. ATEF for riboflavin and PAH are shown before and after addition of tolazoline (Priscoline). Numerals in center represent ATEF ratio Riboflavin/PAH.

Summary. Riboflavin excretion by renal tubular cells would seem to use the transport system for organic bases. This conclusion is based on the fact that tolazoline selectively inhibits riboflavin while not impairing PAH excretion. PAH excretion was even enhanced by tolazoline presumably as a result of diversion of more blood to the portal circulation. Benemid can inhibit transport of both PAH and riboflavin.

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Etiology of the 1927-28 Epidemic of Dengue in Greece. (25474)

M. Theiler, J. Casals and C. Moutousses
Rockefeller Fn. Virus Laboratories, N. Y., and Dept. of Microbiology, National University of Athens, Greece

In 1927 and 1928, an extensive epidemic disease diagnosed as dengue occurred in Greece. It has been estimated that the total number of cases probably exceeded one million(1). The vector was, in all probability, *Aedes aegypti,* which was very prevalent. The diagnosis of dengue was made entirely on clinical grounds, since no satisfactory susceptible experimental animal was available at that time. During recent years, several viral agents have been established in mice from cases diagnosed as dengue. Two of these are known as dengue types I and II(2,3). These viruses are distinct from each other, although there is quite a marked immunological overlap. Investigators in Israel have shown that infections in man with the West Nile virus may produce a disease which clinically resembles dengue(4,5,6). Infections with West Nile virus are exceedingly common in the eastern Mediterranean, not only in Israel but more particularly in Egypt(7). In the latter country the infection is endemic, producing as a rule only a mild febrile disease in young children. Limited serological investigations in these laboratories have not shown infections with either type I or type II dengue in Egypt. The reason for this is probably the scarcity of *Aedes aegypti* in that country. No information concerning the presence of dengue in Israel is known to the authors. The 2 types of dengue and the West Nile virus belong to Casals' group B of immunologically related arthropod-borne viruses. An agent which has recently been isolated in Tanganyika and shown to be the cause of an epidemic, dengue-like disease, is a member of Casals' group A (8,9). These 2 groups of agents are completely distinct immunologically. It was thought of interest to undertake serological investigations with sera obtained from residents of Athens to determine, if possible, the etiology of the "dengue" epidemic which occurred in 1927-28. In these studies, only the possible role of group B agents as the cause of the dengue epidemic was considered.

Materials and methods. In 1958, 56 serum specimens were obtained from Athenian residents ranging in age from 2½ to 70 years. These can be divided into 2 groups. The first consisted of 27 specimens obtained from individuals who were 32 or more years old and consequently were living at the time of the epidemic. The second group consisted of 29 specimens obtained from individuals who, at the time of bleeding, were 29 years of age or younger and thus had been born subsequent to the epidemic. For determining the presence or absence of antibodies, main emphasis was placed on hemagglutination-inhibition (HI) tests(10). All sera were tested against 7 different group B virus antigens: West Nile (strain IG 2266), dengue types I and II, Japanese encephalitis, yellow fever (Asibi), Russian spring-summer encephalitis (RSSE) and SA H 336. The last named virus is an agent closely related to Uganda S(11).

Results. HI tests. The HI antibodies produced following a group B arthropod-borne virus infection are not specific in the sense that only homologous antibodies are developed. Antibodies to related viruses are almost invariably produced, particularly with the passage of time; these antibodies are, as a rule, at lower titers.

In the case of the present sera, the results of the HI tests, as judged by the number of sera positive against each antigen as well as by the relative titers that each serum gave against the different antigens, led to the conclusion that infection with dengue type I was in all probability responsible for the positive reactions. Previous experience with dengue type I infections in rhesus monkeys had indicated clearly that this infection leads to production of HI antibodies capable of reacting in high titer with a wide variety of group B antigens. In the present study, the highest

TABLE I. Results of Hemagglutination-Inhibition Tests.

Age	No. of sera	No. negative	No. positive	% positive
0- 9	14	13	1	
10-19	11	11	0	3
20-29	4	4	0	
30-39	4	2	2	
40-49	5	2	3	67
50+	18	6	12	

titers of HI antibodies were obtained, as a rule, with a dengue type I antigen. In several instances, however, titers obtained with the IG 2266 strain of West Nile virus were higher than those obtained with the dengue type I antigen. Although these results seemed to suggest that some of the infections in Athens might have been due to West Nile virus, it is known from extensive studies on Egyptian sera that the HI pattern following a West Nile infection is characterized by high titers with West Nile and low titers with the 2 strains of dengue, a pattern quite distinct from that observed in the present study.

The results with dengue type I and West Nile antigens indicated that only one of the 29 sera in the younger age group was positive, whereas 17 of 27 (67%) in the older age group were positive. The results are summarized in Table I.

All sera that reacted did so against dengue type I and West Nile antigens, the mean titer with both antigens being 1:80. With 8 sera, the titer was higher against dengue type I; in 5 cases, higher against West Nile, and in 5, the titers were the same.

The HI titers obtained with the type I dengue ranged from 1:10 to 1:1280, whereas the range with the West Nile antigen was from 1:10 to 1:640. Eight units of antigen were used in all cases.

With one exception, all the positive sera gave the highest inhibition with a dengue type I or West Nile antigen. The exception was the serum obtained from an individual born in 1899. This serum reacted in a dilution of 1:80 with a RSSE antigen and in a dilution of only 1:10 with dengue type I and West Nile antigens. This finding suggested that the individual had been infected with RSSE virus.

Neutralization tests. To obtain further information, neutralization tests with dengue type I and West Nile viruses were done. As some of the specimens had been used up, the number of sera which could be tested in this manner was limited. Mixtures of sera and virus were incubated for 1 hour at 37°C before intracerebral inoculation into young adult mice. Titrations of the virus preparations used in the tests indicated that the virus dose per mouse was 40 LD_{50} for the West Nile virus and 50 LD_{50} for the dengue type I. The results are shown in Table II.

All 28 sera in the test were negative in the neutralization test with the West Nile virus, whereas 10 gave clear-cut protection with the dengue type I virus. The one serum in the younger age group that gave a positive reaction in a 1:10 dilution in the HI test with a West Nile but not with any other antigen, failed to exhibit any neutralizing action against either the West Nile or the dengue type I virus. It is possible that the positive reaction obtained with this serum in the HI test is due to a technical error. All 10 sera positive in the dengue neutralization test were also positive in the HI test. One of these had shown a higher HI titer with a West Nile antigen than with a dengue type I antigen. The correspondence between the results

TABLE II. Results of Neutralization Tests.

Results of HI tests	Age group	No. of sera	Dengue type I		West Nile	
			No. negative	No. positive	No. negative	No. positive
Negative	0-29	8	8	0	8	0
	32-70	9	9	0	9	0
Positive	0-29	1	1	0	1	0
	32-70	10	0	10	10	0
Total		28	18	10	28	0

obtained with the HI and the neutralization tests with dengue type I virus was remarkably good.

As mentioned above, the HI studies had indicated the possibility that one of the donors had undergone an infection with RSSE virus. In a neutralization test with RSSE virus, this serum neutralized 300 LD_{50} of the virus, whereas 3 HI negative sera showed no neutralizing action. The donor of this serum had lived all his life in Greece.

These results indicate clearly that the epidemic of dengue in Athens in 1927-28 was, in all probability, due to a dengue type I virus. There is no evidence that any West Nile infections occurred. The high titers obtained in HI tests with a West Nile antigen are possibly the result of the high reactivity of the antigen used. Antigens prepared with the IG 2266 strain of West Nile virus used in these studies have consistently reacted to a higher titer with antisera than antigens prepared from other strains of West Nile.

Discussion. These studies have provided a unique opportunity for acquiring information about the persistence of HI antibodies following a group B arthropod-borne virus infection. In most regions where these agents occur, the infections are endemic, and individuals are exposed to repeated infections by one or more group B agents. In the present instance, however, the complete absence of any group B antibodies in the sera of the younger age group shows that no group B arthropod-borne virus infection has occurred in Athens since the epidemic of 1927-28. Under these circumstances, the close correspondence between the results obtained in the HI and neutralization tests is good evidence that HI antibodies persist for at least 30 years after a dengue type I infection.

Summary. The results of serological investigations on sera obtained from residents of Athens, Greece, indicate clearly that the epidemic of dengue which occurred in that country in 1927-28 was, in all probability, due to type I dengue virus. Antibodies were demonstrated in approximately 70% of sera obtained from persons living at the time of the epidemic. Sera from individuals born subsequent to the epidemic were negative.

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Absorption and Urinary Excretion of Chloramphenicol and 2 Analogues, Thiocymetin and U-15,442 in Normal Men.* (25475)

CALVIN M. KUNIN† AND MAXWELL FINLAND

Thorndike Memorial Laboratory, Second and Fourth (Harvard) Medical Services, Boston City Hospital and Dept. of Medicine, Harvard Medical School, Boston, Mass.

Following the discovery of chloramphenicol and elucidation of its chemical structure, numerous derivatives were prepared in different laboratories. However, in the various test systems employed, none of them have shown

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† Present address: Univ. of Virginia College of Medicine, Charlottesville.

antimicrobial activity as great as that of the parent compound(1), nor have any thus far proved to be as useful therapeutically. The possibility remains that certain of the chloramphenicol derivatives, by virtue of some special pharmacologic property, may be more desirable than chloramphenicol as a therapeutic agent in man. Chloramphenicol has been shown to be rapidly inactivated *in vivo* by conjugation with glucuronic acid, and this largely accounts for the recovery in urine of man of only 5-15% of microbially active, unconjugated drug(2,3,4). Conceivably a weaker derivative which is inactivated much more slowly than the parent compound *in vivo* may produce an equivalent or even greater amount of antimicrobial activity in blood, tissue and urine. With this in view, an investigation of absorption and urinary excretion of 2 chloramphenicol derivatives that had good activity *in vitro* was undertaken in man. Both of these compounds differ from the parent drug only in substitutions for the nitrophenyl group, one derivative, Thiocymetin® (Win 5063-2), substituting 4-methylsulfonylphenyl, the other U-15,442 (Win 5094-2), substituting 4-methylmercaptophenyl.

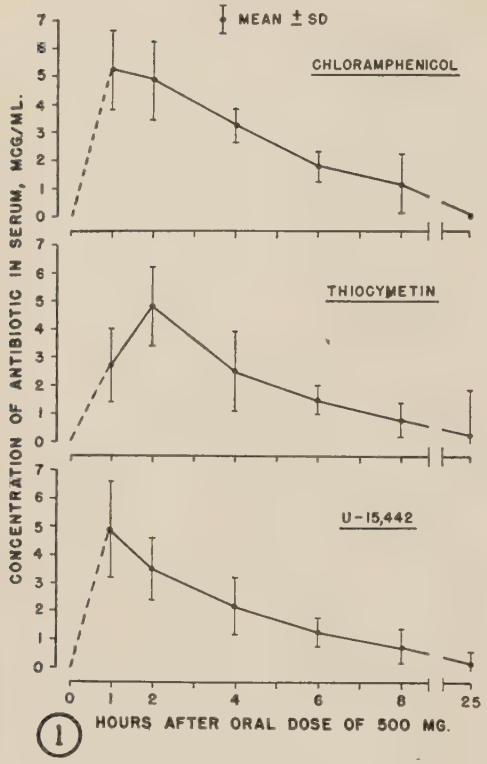
Materials and methods. An oral dose of 500 mg of chloramphenicol, Thiocymetin and U-15,442§ was given, according to a Latin square design, in rotation to 6 healthy young men. Doses were separated by at least one week. On each test day the subjects were in the fasting state except for liberal water intake. Venous blood samples were obtained prior to and at 1, 2, 4, 6, 8 and 25 hours after the dose. The first meal was taken after the 4-hr specimen. Serums were separated as soon as possible and stored at –20°C until time of assay. All urine passed during 48 hours following the dose was collected in sterile containers and immediately refrigerated; aliquots from pools obtained at 0-4, 4-8, 8-12, 12-24 and 24-48 hours were kept frozen at –20°C until time of assay. Specimens of serum and urine were assayed by a modified cup-plate method employing *Pasteurella bovisepticus* as the test organism and levels were expressed in μg/ml based on comparison with standard drug corresponding to the one administered. These tests were done in the Research Laboratories, Upjohn Co. (to be referred to as Laboratory A) to which the specimens were delivered in the frozen state. Urines were also assayed in this laboratory (hereafter referred to as Laboratory B) using the standard *Sarcina lutea* cup-plate method described for chloramphenicol(5), and employing standards of all 3 drugs simultaneously with each test so that results could be expressed in terms of activity equivalent of each drug. The assay strain of *P. bovisepticus* was inhibited by 0.19, 0.09 and 0.09 μg/ml of chloramphenicol, Thiocymetin and U-15,442, respectively. The strain of *S. lutea* was less sensitive but otherwise satisfactory; it was inhibited by 3, 10, and 15 μg/ml of chloramphenicol, Thiocymetin and U-15,442, respectively.

Results. Serum levels. Average concentrations of the 3 analogues in serum of 6 subjects are shown in Fig. 1. Average peak levels were nearly the same for the 3 analogues but were achieved later with Thiocymetin than with the other 2. At 6 hours, average concentrations of all 3 drugs were again about the same. Slight activity of Thiocymetin and U-15,442 was still measurable 25 hours after these doses, but none was detectable after the dose of chloramphenicol.

Urinary excretion. Average amounts of the 3 drugs recovered in urine, as determined in each laboratory, are given in Table I. Values obtained in Lab. A were slightly lower than those obtained in Lab. B for chloramphenicol and U-15,442, but for Thiocymetin they were about one-third lower. Despite these differences the patterns of the 48 hour cumulative excretion of the 3 drugs as determined in the 2 laboratories were quite similar (Fig. 2). In both studies considerably more thiocymetin was recovered than U-15,442; recoveries of chloramphenicol in urine were lowest of the 3.

The data presented in Fig. 3 permit comparison of relative antimicrobial activity of each analogue in urine, expressed in chloramphenicol "equivalents" as determined in the *S. lutea* method. Thiocymetin, in each sequential time period, and over the total pe-

§ Kindly supplied in coded capsules by Upjohn Co. from bulk material obtained from Winthrop Laboratories; the generic name of Thiocymetin is dextrosulphenidal.



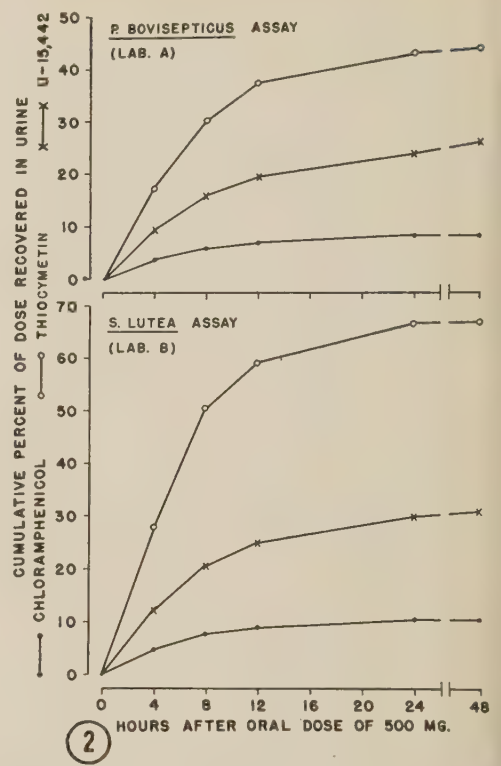
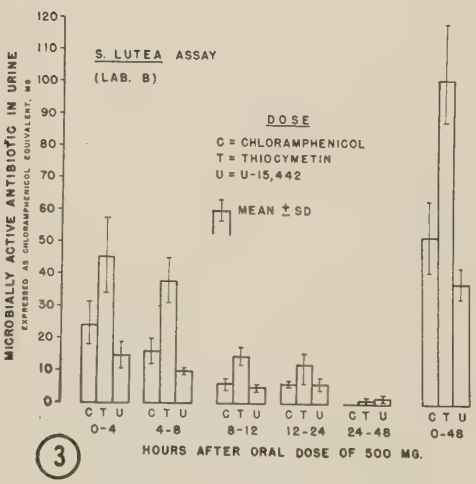


FIG. 1. Avg concentrations of chloramphenicol and 2 analogues in serum of same 6 subjects after single oral doses of 500 mg.

FIG. 2. Avg excretion of microbially active chloramphenicol and 2 analogues in urine of 6 subjects after single oral doses of 500 mg as determined in 2 laboratories.

FIG. 3. Avg excretion of active chloramphenicol and 2 analogues, each calculated as chloramphenicol equivalents, after single oral doses of 500 mg.



riod of 48 hours, produced significantly more antimicrobial activity in urine than did either chloramphenicol or U-15,442. The same relationships hold for results expressed in terms of the other 2 drugs; only the heights of all the bars are different.

Discussion. These data indicate that levels of microbially active thiocymetin and U-15,442 produced in serum during the first few hours after an oral dose are quite comparable to those obtained with chloramphenicol; however, peak levls were achieved somewhat later with Thiocymetin and activity was no longer detectable in serum 25 hours after the dose of chloramphenicol, but was still measurable in some subjects after doses of the other 2 ana-

TABLE I. Recovery of Chloramphenicol and 2 Analogues in Urine of 6 Healthy Young Men after a Single Oral Dose of 500 mg.

Hr after dose	Urinary excretion: Chloramphenicol mg ± S.D.	% *	Thiocymetin mg ± S.D.	%	U-15,442 mg ± S.D.	%
	Laboratory A, *P. bovisepticus* assay					
0- 4	18.0 ± 6.4	3.6	87.1 ± 22.6	17.4	47.5 ± 12.8	9.5
4- 8	12.3 ± 2.9	2.4	65.0 ± 10.6	13.0	31.6 ± 3.7	6.3
8-12	5.3 ± 1.6	1.0	33.9 ± 7.3	6.7	18.1 ± 8.2	3.6
12-24	5.5 ± 1.8	1.1	28.4 ± 8.9	5.6	23.3 ± 4.4	4.6
24-48	.8 ± .5	.1	5.6 ± 5.4	1.1	8.6 ± 5.5	1.7
Total	41.7 ± 9.2	8.2	220.0 ± 28.5	43.8	129.1 ± 24.9	25.7
	Laboratory B, *S. lutea* assay					
0- 4	23.8 ± 6.8	4.7	140.3 ± 35.5	28.0	61.3 ± 17.1	12.2
4- 8	16.2 ± 3.7	3.2	112.5 ± 26.4	22.5	41.6 ± 4.1	8.3
8-12	5.9 ± 1.5	1.1	44.4 ± 7.7	8.8	21.6 ± 3.9	4.3
12-24	5.9 ± 3.2	1.1	35.5 ± 11.4	7.1	25.8 ± 8.0	5.1
24-48	†	—	2.7 ± 5.2	.5	5.4 ± 4.2	1.0
Total	51.7 ±11.0	10.1	335.7 ± 41.5	66.9	155.5 ± 21.0	30.9

Each value shown is the mean for the same 6 subjects, expressed as activity of drug ingested.
* % of administered oral dose. † Undetectable.

logues. Recovery of the active drug in urine was several times as great after Thiocymetin and significantly greater after U-15,442 than after chloramphenicol, when expressed as percent of dose ingested. However, when expressed in the equivalent of chloramphenicol activity, the dose of Thiocymetin still yielded significantly more activity in urine than a similar dose of chloramphenicol, but the advantage of U-15,442 was lost due to the lesser activity of the latter against the assay organism.

From the therapeutic point of view at least 2 factors other than absorption, excretion and inactivation must be considered in comparing these 3 drugs, namely their relative *in vitro* activity against human pathogenic bacteria and their relative toxicity. Since chloramphenicol has been widely used recently in treatment of infections with staphylococci that are resistant to other antibiotics, a number of recently isolated strains were tested for their susceptibility to these 3 agents by a 2-fold dilution method on agar plates. The results (Table II), indicate that chloramphenicol is the more active agent, on the average at least twice as active as the other two. Since blood levels over an 8-hour period after a dose in normal individuals were at least as high with chloramphenicol as with the other derivatives, the systemic antistaphylococcal effect may be expected to be greater. The activity recoverable in urine, on the other hand, was greater with Thiocymetin even when expressed as active chloramphenicol equivalents, so that the former might be considered advantageous in treatment of urinary tract infections. The results do indicate, however, that Thiocymetin and U-15,442, particularly the former, are inactivated more slowly or by different mechanisms than chloramphenicol. The overriding consideration from the therapeutic point of view is the much greater toxicity of Thiocymetin as revealed both in laboratory animals and in preliminary clinical trials which preclude its use (personal communication). Nevertheless, these findings indicate that further search for chloramphenicol derivatives that are less toxic and also inactivated more slowly or to a lesser degree than chloramphenicol, may be worthwhile.

TABLE II. Susceptibility of 178 Recently Isolated Strains of *Staphylococcus aureus* to Chloramphenicol and 2 of Its Analogues.

Minimum inhibiting conc., μg/ml	Chloramphenicol	Thiocymetin	U-15,442
	% of strains		
6.3	12.9		
12.5	55.1	4.5	5.1
25	23.6	47.8	83.1
50	1.7	37.6	6.7
100	5.1	1.1	2.8
>100	1.7	9.0	2.2

Summary. Comparison of absorption and urinary recovery of chloramphenicol and 2 of its derivatives, Thiocymetin and U-15,442, has been carried out following administration of single oral doses of 500 mg each in 6 healthy human volunteers. Similar peak concentrations of these drugs were noted in serum but they were achieved later after the dose of Thiocymetin. At 25 hours slight antibiotic activity was still discernible with the 2 derivatives, but not with chloramphenicol. In view of the superior antimicrobial activity of chloramphenicol the 2 derivatives are considered to be inferior to the parent compound in producing antimicrobial activity in serum. In contrast, much higher urinary recoveries were achieved with the 2 derivatives than with chloramphenicol. In the case of Thiocymetin urinary recovery was so great as to more than offset its inferior antimicrobial activity; this was not the case with U-15,442. The high recovery of active Thiomycetin in urine suggests that it is inactivated more slowly or by a different mechanism than is chloramphenicol.

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A Simple Chromatographic Method for Preparation of Gamma Globulin.* (25476)

HILTON B. LEVY AND HERBERT A. SOBER
U. S. Dept. of H.E.W., N.I.H., Nat. Inst. of Allergy and Infectious Diseases, Nat. Cancer Inst., Bethesda, Md.

Many serological procedures might be improved by use of gamma globulin rather than whole serum as the source of antibody. For example, non-specific inhibitors of viruses might be removed in some cases where antiviral sera are being studied. Furthermore, in work with fluorescent antibody, non-specific staining might be reduced if gamma globulin fractions were used instead of serum. Most methods for preparation of gamma globulin are not convenient for use by the usual virology or immunology laboratory. The high capacity of the cellulose ion exchange adsorbents(1), and the relatively mild conditions of salt and pH required for adsorption and elution have suggested their use in such preparation(2). A slight modification of the chromatographic procedure described by Sober and Peterson(3) is presented here which allows preparation of gamma globulin in a stepwise procedure, free from macroglobulins, high molecular weight serum protein with gamma globulin mobility, and non gamma globulin serum proteins.

Methods. Ion exchange adsorbents. The anion exchanger, diethylamino-ethylcellulose (DEAE), (40-100 mesh, 0.96 meq/g) was prepared by the method of Peterson and Sober(1), or purchased. It was washed with 0.5 N NaOH 3 times to remove color and to regenerate the column, then suspended in water and washed to neutrality. The slurry was adjusted to pH 6.3 by adding 0.2 M NaH_2PO_4, and washed several times with the starting buffer (0.0175 M phosphate (Na^+), pH 6.3). A large batch of the adsorbent may be so prepared and stored in the refrigerator.

* We express our appreciation to Drs. Katherine Cook, N. I. H., Pekka Halonen, Univ. of Helsinki, and Major E. L. Buescher, Walter Reed Army Inst. of Research for generous help in some biological assays.

Before use it is resuspended gently, and the slowly sedimenting "fines" discarded as they unduly interfere with the flow rate of the column. The cation exchanger, carboxymethylcellulose (CM) (100-230 mesh, 0.74 meq/g) was synthesized as described(1). It was prepared for use by washing 3 times with 0.5 M NaCl—0.5 M NaOH, then with water till neutral, then with 0.0175 M PO_4 pH 6.3, till the pH of the wash was 6.3. *Antibody assay.* Adenovirus antibodies were assayed according to procedures given by Rowe *et al.*(4). ECHO virus antibodies as well as antibody to the tissue on which the viruses were grown were assayed according to Halonen(5). Antibodies to diphtheria and tetanus toxoids were determined by hemagglutination technics, using serial dilution of serum against erythrocytes sensitized by the method of Landy *et al.*(6), using as a standard a modified antitoxin of known unitage. Neutralizing antibodies to Russian Spring-Summer Encephalitis were estimated by the method of Rivers *et al.*(7). *Separation procedure.* (1) The serum to be fractionated is dialyzed overnight *vs.* 100-200 volumes of 0.0175 M PO_4 buffer (Na^+), pH 6.3 in the cold. The slight precipitate formed may be removed by centrifugation or may be retained and put on the column. (2) For 2 ml of the dialyzed serum, a column of DEAE-cellulose is prepared of about 1 cm diameter and 5 cm height, using pressure, not to exceed about 10 lb/sq in, preferably in the form of nitrogen. The column should not be allowed to dry out during the preparation. The column is then washed with about 50 ml more of the starting buffer. (3) With the liquid level of the starting PO_4 buffer just at the top of the adsorbent, the serum is carefully added and pushed into the column with gentle gas pressure. The gamma globulin, the only serum protein that is not adsorbed under the indicated conditions, is washed through with more of the pH 6.3 buffer. For 2 ml of serum about 6 ml of buffer will remove most of the gamma globulin, but about 20 ml is needed for complete recovery. The macroglobulin remains on the column with the other non-gamma globulin serum proteins and may be removed from the column by washing with 2 M NaCl in 0.4 M Na-phosphate, pH 5.2.

TABLE I. List of Antisera Fractionated and Tested.

Immunizing agent	Species of serum	Test method	% antibody recovered in: Gamma globulin	% antibody recovered in: Remainder
Adenovirus, Type 2	Rabbit	Comp. fix.	50	0
Idem	"	Neut.	100	0
ECHO 6	Guinea pig	Comp. fix.	80	0
Monkey kidney t.c.	"	*Idem*	80	0
ECHO 6	Rabbit	"	80	0
" 14	"	"	40	0
Tetanus toxoid	Human (2)	HA	100	0
Diphtheria toxoid	"	"	50	0
Russian S-S enceph.*	"	Neut.	300	0

* Russian Spring-Summer Encephalitis.

Results. The systems to which the procedure has been successfully applied are given in Table I. It should be realized that reproducibility of some of the above testing systems is no better than plus or minus 2-fold. Therefore the results of Table I indicate that the described fractionation procedure separates a non-adsorbed chromatographic fraction, which contains only gamma globulin and which accounts for all the antibody. All the gamma globulin fractions were examined qualitatively in the Spinco paper electrophoresis apparatus, or in the Aminco-Stern boundary electrophoresis apparatus in veronal buffer at pH 8.6 and some were analyzed quantitatively. All samples proved to be gamma globulin either without detectable contamination or contaminated only with a small amount of β-globulin. The "adsorbed-fraction," that was eluted from the column by the higher salt concentration, showed the other serum components with the small amount of gamma globulin that could be attributed to the macroglobulin component.

In addition to antisera mentioned in Table I, 3 samples of horse serum that showed anti-type I poliovirus activity were fractionated by the present method. All the anti-poliovirus activity was found in the non-adsorbed gam-

ma globulin fraction. For several reasons this material has not been considered as an antibody(8).

It should be noted that the present method dilutes the gamma globulin 4-10 fold. If this dilution should be undesirable, the gamma-globulin eluate may be applied directly to a carboxymethylcellulose (CM) column, equilibrated with pH 6.3, 0.0175 M PO_4 buffer onto which the gamma gobulin will be adsorbed(2, 3). It may be then eluted in a more concentrated form by 2 M NaCl in 0.05 M Na-phosphate pH 6.9. Any other concentration method may, of course, be used.

Summary. A relatively simple method for separation of gamma globulin from serum has been presented. The procedure has been applied to a number of antisera, and has been shown with them at least to give reasonably good localization of antibody in the gamma globulin fraction.

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Effect of Pluronic F68 on Growth of Fibroblasts in Suspension on Rotary Shaker. (25477)

H. E. SWIM* AND R. F. PARKER

Dept. of Microbiology, Western Reserve University, Cleveland, O.

Recent investigations have established the feasibility of propagating certain strains of mammalian cells in suspension, and a number of technics have been described. These include the tumble tube of Owens *et al.*(1), the rotary shaker of Earle *et al.*(2), the roller tube of Graham and Siminovitch(3), the suspended stirrer (spinner culture) of Cherry and Hull(4), the fermentor of McLimans *et al.*(5), and a variety of modifications of the foregoing(6,7,8,9,10). Use of conventional rotary shakers to provide agitation eliminates the need for special equipment and the method is particularly suitable for experiments involving many cultures of relatively large volume. Formation of a non-cellular precipitate(11) under certain conditions, however, is a major drawback of the shaker method. In media containing serum, the precipitate is presumably protein in nature(11,12) and its formation is related to strain of cells and environmental conditions employed(11). Precipitation of constituents of the medium not only results in erratic growth of cells but imposes severe limitations on their use in studies of metabolism. This report describes the use of Pluronic F68 in shake cultures as a means of essentially eliminating the precipitate formed in a medium containing horse serum.

Materials and methods. The Pluronics† are blockpolymer surfactants prepared by adding ethylene oxide to both ends of a polyoxypropylene polymer. Pluronic F68, hereafter referred to as F68, is a solid with a molecular weight of approximately 8750. About 80% of the molecule, by weight, consists of hydophilic polyoxyethylene groups and the remainder of hydrophobic polyoxypropylene groups. Stock cultures of strain U12-29 fi-

* Research Fellow, Am. Cancer Soc., Cuyahoga County Unit. Aided by grant from U.S.P.H.S.

† Authors are indebted to M. J. Conway, Wyandotte Chemicals Corp. for generous supply of F68 and other Pluronics.

broblasts(13) were propagated continuously in Bellco-type spinner flasks‡ in medium 29 composed of S103(14) fortified with 20% (v/v) horse serum (NHS). The flasks were stoppered and contained ½ to ⅔ their volume of medium. Cells were maintained in pseudo exponential growth by replacing a portion of the suspension with fresh medium at appropriate intervals. Aqueous solutions containing 5 to 25% F68 were sterilized by filtration through Selas§ candles of 02 porosity. All glassware in both spinner and shake cultures was siliconized with saturated solution of silicone‖ in carbon tetrachloride. After draining, the flasks were heated at 160°C for 45 minutes. Experiments with shake cultures were performed as follows: Aliquots of cell suspension from spinner cultures were centrifuged and the supernatant discarded. The cells were suspended in experimental medium at concentration of 0.3 to 0.6 × 10^5 cells/ml and 75 ml was added to each of a series of 125-ml Florence flasks. The flasks were gassed (45% O_2, 5% CO_2 and 45% N_2), stoppered, and incubated at 37°C on a rotary shaker¶ at 200 rpm. Samples were removed at 24 to 72 hour intervals and the cells enumerated by the citric acid procedure employed in this laboratory(15).

Results. When medium 29 was shaken at 37°C in absence of cells, precipitation was evident within a few hours and was usually complete in 24 to 48 hours as judged by volume of pellet obtained upon centrifugation of aliquots of the medium at 1000 × G for 15 minutes. Media prepared with 10 different lots of serum all yielded considerable precipitate. The quantity and general appearance of the precipitate varied with the lot of serum employed. Some media became opalescent and considerable amorphous debris accumulated, whereas in others a granular precipitate was formed. When medium 29 was supplemented with 0.05 to 5% F68, precipitation of components of the medium was virtually eliminated. At these concentrations of F68 most sera remained clear for the experimental period of 7 to 10 days. In a few instances a small amount of granular-type precipitate was evident after 2 to 3 days, but this did not increase appreciably on continued shaking. With media prepared from a limited number of sera, F68 was effective in either preventing or markedly reducing precipitation in concentrations as low as 0.01%. The results of preliminary experiments indicate that F68 is also effective in reducing precipitation in similar media prepared from human serum.

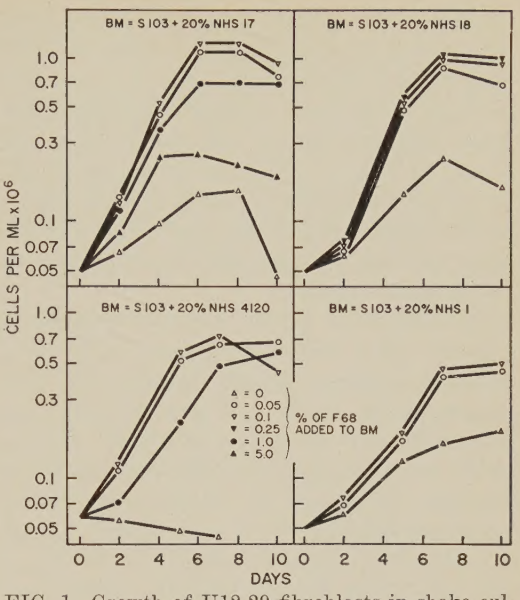

FIG. 1. Growth of U12-29 fibroblasts in shake cultures as a function of concentration of Pluronic F68 in medium 29. Each point on curves represents avg values for 2 or more experiments. BM refers to basal medium to which F68 and horse serum was added.

The effect on proliferation of U12-29 fibroblasts in shake cultures of adding F68 to basal medium is illustrated in Fig. 1. Amount of growth in absence of F68 is consistently less than one half that obtained when the Pluronic is added to the medium. The results varied with the lot of serum employed. For example, some growth was obtained in absence of F68 in media prepared with either NHS 17, 18 or 1, whereas the cells began to degenerate in a few days in a medium con-

‡ Flasks manufactured by Bellco Glass, Vineland, N. J.

§ Porcelain filters manufactured by Selas Corp., Philadelphia, Pa.

‖ A silicone lubricant manufactured by Dow Corning Corp., Midland, Mich.

¶ Model S-3 shaker manufactured by Brunswick Scientific Co., New Brunswick, N. J.

taining NHS 4120. Likewise, significantly less growth was obtained in presence of F68 with NHS 1 than with the other 3 sera. Differences of the same order of magnitude have been observed among additional sera not shown in Fig. 1. Variations in ability of different sera to promote growth are not unique to the shaker system since individual lots exhibit the same relative activity in stationary and spinner cultures.

A subline of strain U12-29, designated U12-79, grows well in stationary flasks in a medium composed of S103 fortified with 5% dialysed horse serum, but fails to proliferate in either shake or spinner cultures. Bryant *et al.*(16) observed that addition of the lower viscosity grades of the substituted cellulose polymer, Methocel** permits growth of the NCTC 2071 strain of fibroblasts on the shaker in a chemically defined medium. In the absence of Methocel the cells degenerate when shaken but proliferate in the same medium in stationary flasks. The results obtained with U12-79 indicate that F68 does not function as does Methocel in facilitating growth of cells in minimal media in shake cultures. The growth enhancing activity of F68 in shake cultures appears to be directly related to its ability to prevent precipitation of protein from the medium. Thus, growth curves of U12-29 in shake cultures containing low levels of F68 (Fig. 1) are indistinguishable from those obtained in a Pluronic-free medium in spinner flasks where precipitation is minimal.

Summary. A medium composed of the defined solution S103 supplemented with 20% horse serum yields a noncellular precipitate when agitated on a rotary shaker. This precipitation results in loss of from 25 to 100% of growth-promoting activity of the medium for strain U12-29 fibroblasts in shake cultures. Addition of the polyglycol, Pluronic F68, to the medium largely eliminates precipitation. Under these conditions, growth of U12-29 in suspension on a rotary shaker is comparable to that obtained in spinner cultures.

** Manufactured by Dow Chemical Co., Midland, Mich.

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Effect of Environmental Temperature upon Cholesterol Induced Aortic Atherosclerosis in the Rabbit. (25478)

W. A. Sodeman and John T. Logue
Jefferson Medical College, Philadelphia, Pa.

Hypercholesterolemia and aortic atherosclerosis resulting from cholesterol feeding in rabbits is a well established procedure. Administration of high protein diets(1), alloxan, (2,3), detergents(4), heparin(5), hyaluronidase(6), cholestanol(7), testosterone and estradiol(8), potassium thiocyanate(9), and cortisone(10,11) as well as production of concomitant malnutrition(12), have all been shown to vary the level of blood cholesterol and alter the degree of resulting atherosclerosis in rabbits fed a high cholesterol diet. Similar studies relating degree of hypercholesterolemia and extent of atherosclerosis to age and weight of experimental animals have been reported(13). It is apparent from these studies that degree of hypercholesterolemia is not the sole factor in determining extent of atherosclerotic aortic lesions in the rabbit. Indeed often more marked sclerosis has been demonstrated in those animals with lower serum cholesterol values. A satisfactory explanation of this phenomenon is not apparent. Certainly, the aortic intima varies in its ability to withstand or metabolize plasma lipid loads under varying conditions studied. Seasonal variations in incidence and severity of atherosclerotic complications is a frequently observed clinical phenomenon. Indeed many years ago Mills(14) suggested that variations in climate have a direct effect on death rates from coronary artery disease. Whether environmental temperature itself may induce changes in ability of intimal cells to respond to hypercholesterolemia has not been settled. With this question in mind, we carried out the following experiment using environmental temperature as a variable while attempting to keep other conditions constant. For this experiment we were kindly granted the use of climatic laboratory at the College of Agriculture of Missouri.

Materials and methods. Sixteen 6-month-old, male, albino rabbits were divided into 2 groups. One group was maintained at environmental temperature of 45-50°F, while the other group was kept at 80-85°F. Relative humidity of both groups was maintained at 60-65%. Two rabbits in each group served as controls and ate a measured standard diet of Purina rabbit pellets with water *ad lib.* After 2 weeks, in which food intake and weight gain of both groups were comparable, cholesterol enriched rabbit pellets were substituted in both groups excluding controls. The cholesterol chow was prepared following the method of Wang *et al.*(15). Amorphous pure cholesterol U.S.P. (Merck & Co.) was dissolved in 10% solution of ether and sprayed over thin layers of Purina pellets. After the ether evaporated the cholesterol covered and infiltrated the pellets as a fine film. Average daily intake of cholesterol/rabbit was approximately 1 gram. On the first, 21st, and 42nd day average serum cholesterol levels were determined, following the method of

TABLE I. Average Serum Cholesterol Values for Rabbits Maintained at 45-50°F and 80-85°F.

No. of days fed cholesterol⟶	1st day	21st day	42nd day
Cholesterol fed rabbits at 45-50°F			
Total cholesterol	113.8	1034.1	2073.8
Free "	31.1	372.6	847.8
Esters	82.7	661.5	1226.0
Ester %	72.7	64.0	59.1
Cholesterol fed rabbits at 80-85°F			
Total cholesterol	104.5	1524.8	2632.3
Free "	40.9	672.3	892.8
Esters	63.6	852.5	1739.5
Ester %	60.9	55.9	66.1
Values for rabbits on normal diet after 21 days			
Control rabbits at 45-50°F			
Total cholesterol		102.2	
Free "		22.4	
Esters		79.8	
Ester %		78.0	
Control rabbits at 80-85°F			
Total cholesterol		111.0	
Free "		34.8	
Esters		76.3	
Ester %		69.8	

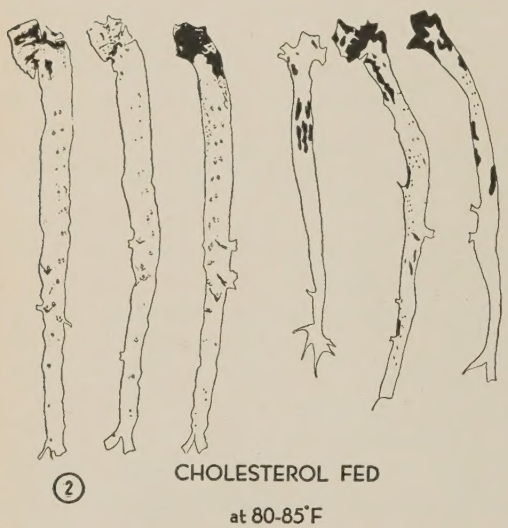

FIG. 1 and 2. Ink drawings showing distribution of aortic sclerotic plaques in rabbits fed cholesterol and maintained at 45-50°F (Fig. 1) and 80-85°F (Fig. 2).

Schoenheimer and Sperry(16) (See Table). On forty-fifth day the animals were sacrificed. Aortas were dissected free of excess fat and fixed in 10% formalin solution for several days, then stained with Sudan IV (usually 2 to 3 minutes), and decolorized with 70% alcohol until good photographic contrast was obtained. From photographs of the mounted aortas, ink drawings were prepared showing distribution of aortic sclerotic plaques. Two rabbits in the 45-50° group and one rabbit in the 80-85° group died shortly after start of experiment and were excluded. One additional rabbit in the 80-85 degree group died following a convulsion on thirtieth day and was included.

Results showed remarkable reduction in plaque formation in animals kept at higher temperature levels in spite of higher total cholesterol and ester values in this group (Fig. 1 and 2). Electrophoretic patterns of sera showed a higher, sharper peak in the beta range in animals kept at higher temperature. Radioactive iodine uptake studies did not reveal any significant difference in the 2 groups.

Summary. The findings indicate that heat stress in rabbits raised under conditions studied, produced a profound effect upon extent and distribution of aortic atherosclerotic plaque formation.

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